ผลต่อต้านเชื้อก่อโรคในผึ้งโดยแบคทีเรียในทางเดินอาหารของผึ้งโพรง Apis cerana

นางสาวปวรรัตน์ นนทภา

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ANTAGONISTIC EFFECT AGAINST BEE PATHOGENS BY GUT BACTERIA IN CAVITY NESTING HONEYBEE Apis cerana

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ปวรรัตน์ นนทภา : ผลต่อต้านเชื้อก่อโรคในผึ้งโดยแบคทีเรียในทางเดินอาหารของผึ้งโพรง Apis cerana. (ANTAGONISTIC EFFECT AGAINST BEE PATHOGENS BY GUT BACTERIA IN CAVITY NESTING HONEYBEE Apis cerana) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.จันทร์เพ็ญ จันทร์เจ้า, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.อรวรรณ ดวงภักดี, 114 หน้า.

ในฟาร์มผึ้ง มักพบปัญหาเกี่ยวกับศัตรูและโรคในผึ้ง อันนำไปสู่การสูญเสียทางเศรษฐกิจในอุตสาหกรรมการเลี้ยงผึ้ง ยกตัวอย่างเช่น โรคตัวอ่อนเน่าอเมริกัน (AFB) ที่เกิดจากเชื้อ Paenibacillus larvae, โรคโนซีม่าเกิดจากเชื้อ Nosema ceranae และ N. apis, โรคแซคบรูด (SBV) เกิดจากเชื้อ sacbrood bee virus (SBV), และโรคชอล์คบรูดที่เกิดจากเชื้อ Ascosphaera apis ซึ่งในงานวิจัยนี้ได้ทำการสำรวจการติดเชื้อ P. larvae, N. ceranae, N. apis, และ A. apis ในผึ้งโพรง 50 รังที่เก็บจากฟาร์มเลี้ยงผึ้ง 10 แหล่งในจังหวัดสมุทรสงครามและชุมพร ซึ่งอยู่ทางภาคกลางและภาคใต้ของประเทศไทย ทำการสกัดดีเอ็นเอจากตัวหนอนดักแด้และตัวเต็มวัยผึ้ง จากนั้นทำการเพิ่มปริมาณดีเอ็นเอโดยวิธี multiplex PCR ซึ่งในแต่ละปฏิกิริยาประกอบด้วยไพรเมอร์สองคู่ (16S rRNA ของ P. larvae และ cytochrome b ของ A. cerana), ไพรเมอร์สามคู่ (16S rRNA ของ N. ceranae, N. apis และ RpS5 ของ A. mellifera), ไพรเมอร์สองคู่ (pol ของ SBV และ 28S rRNA ของ A. mellifera), และไพรเมอร์สองคู่ (5.8S rRNA ของ A. apis และ RpS5 ของ A. mellifera) ผลการศึกษาพบตัวอย่างจำนวน 80%, 94%, 96%, และ 80% ไม่มีการติดเชื้อ P. larvae, N. ceranae, N. apis, และ A. apis ตามลำดับ เพื่อสร้างแผนภูมิทางสายวิวัฒนาการของ P. larvae, N. ceranae, N. apis, และ A. apis จากลำดับเบสบางส่วนของ 16S rRNA สามารถแยก cluster ได้จำนวน 6, 2, 1, และ 4 clusters ที่แตกต่างกัน ตามลำดับ นอกจากนี้ยังทำการจัดจำแนกแบคทีเรียจากลำไส้ของผึ้งที่มีสุขภาพดีโดยใช้วิธีทดสอบการหมักพบแบคทีเรียในสกุล Bifidobacterium และ Lactobacillus และผลจากการวิเคราะห์ลำดับเบสบางส่วนของ 16S rRNA สามารถจัดจำแนกแบคทีเรียในสกุล Bacillus, Lactobacillus, และ lactic acid bacteria จากนั้นนำแบคทีเรียที่จำแนกได้มายับยั้งเชื้อ P. larvae สายพันธุ์ 01 และ 02 โดยใช้วิธี agar well diffusion assay พบว่าแบคทีเรียในสกุล Bacillus, Pantoea, Azotobacter, Klebsiella, และ Lactobacillus มีผลต่อต้านเชื้อก่อโรคได้ดี และพบว่า Azotobacter มีผลต่อต้านสูงสุดต่อสายพันธุ์ 02 มีความสัมพันธ์ของเส้นผ่านศูนย์กลางการยับยั้งที่ 56.68c±3.17% นอกจากนี้ได้นำส่วนใสจากการปั่นเหวี่ยงตะกอนออกของ Bacillus, Lactobacillus, Klebsiella, Azotobacter หลังบ่มที่อุณหภูมิ 37 ºC และส่วนใสของ Lactobacillus, Klebsiella, Azotobacter, Bacillus หลังบ่มที่อุณหภูมิ 90 ºC มาทำการทดสอบ พบผลต่อต้านสายพันธุ์ 01 และ 02 แต่ไม่พบผลต่อต้านเชื้อเมื่อบ่มส่วนใสที่ 121 ºC, และบ่มร่วมกับ proteinase K และ RNase A นอกจากนี้เมื่อปรับส่วนใสของเชื้อ Pantoea ให้มีค่า pH 5, Bacillus ให้มีค่า pH 3, Klebsiella ให้มีค่า pH 3, Lactobacillus ให้มีค่า pH 8 และ 10, และ Klebsiella ให้มีค่า pH 10 พบว่าให้ผลต่อต้านสายพันธุ์ 01 และ 02 สูงสุดจากการวิเคราะห์ความแตกต่างของค่าเฉลี่ยอย่างมีนัยสำคัญทางสถิติที่ระดับความเชื่อมั่น 95 เปอร์เซ็นต์ ผลต่อต้านจากส่วนใสที่ภาวะ pH 8-10 มีฤทธิ์ลดลงเมื่อเปรียบเทียบกับส่วนใสที่ภาวะ pH 5 ซึ่งมีค่า pH ใกล้เคียงกับภาวะกรดอ่อน ๆ ในท้องผึ้ง และส่วนใสที่อุณหภูมิ 90 ºC มีผลต่อต้านสายพันธุ์ 01 และ 02 สูงกว่าส่วนใสที่อุณหภูมิ 37 ºC จึงสามารถสรุปได้ว่าพบแบคทีเรียในทางเดินอาหารที่สามารถต่อต้านการเติบโตของเชื้อ P. larvae ได้

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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PAWORNRAT NONTHAPA: ANTAGONISTIC EFFECT AGAINST BEE PATHOGENS BY GUT BACTERIA IN CAVITY NESTING HONEYBEE APIS CERANA. ADVISOR: ASSOC. PROF. CHANPEN CHANCHAO, Ph.D., CO-ADVISOR: ASST. PROF. ORAWAN DUANGPHAKDEE, Ph.D., 114 pp.

In an apiary, honeybees have had problems with pests and diseases leading to economic loss in apiculture. For example, American foulbrood (AFB) disease was caused by Paenibacillus larvae. Nosema disease was caused by Nosema ceranae and N. apis. Sacbrood disease was caused by sacbrood bee virus (SBV) and chalkbrood disease was caused by Ascosphaera apis. In this research, infection of P. larvae, N. ceranae, N. apis, SBV, and A. apis were investigated in Apis cerana. Fifty colonies were collected from ten apiaries in Samut-songkhram and Chumphon provinces in the central and the south of Thailand, respectively. Total DNA was extracted from larvae, pupae, and adults and amplified by multiplex PCR. In each reaction, it comprised of two pairs of primers (16S rRNA of P. larvae and cytochrome b of A. cerana), three pairs of primers (16S rRNA of N. ceranae, of N. apis, and RpS5 of A. mellifera), two pairs of primers (pol of SBV and 28S rRNA of A. mellifera), and two pairs of primers (5.8S rRNA of A. apis and RpS5 of A. mellifera). The results showed that 80%, 94%, 96%, and 80% of samples were not infected by P. larvae, N. ceranae, N. apis, and A. apis, respectively. The phylogenetic lineage depending on the partial sequences of 16S rRNA of P. larvae, N. ceranae, N. apis, and 5.8S rRNA of A. apis exhibited six, two, single, and four distinct clusters, respectively. Bacteria in healthy bee's gut such as Bifidobacterium sp., Lactobacillus sp. were identified by fermentation test. Bacillus sp., Lactobacillus sp., and lactic acid bacteria were identified by the analyzed sequence of 16S rRNA. Then, the antagonistic effect of selected gut bacteria which were Bacillus sp., Pantoea sp., Azotobacter sp., Klebsiella sp., and Lactobacillus sp. against P. larvae, strain 01 and 02 was determined by agar well diffusion assay. It revealed that Azotobacter sp. showed the highest relative inhibitory zone effect on strain 02 with 56.68c±3.17% in diameter. The supernatant of targeted bacteria showing the antibacterial activities against strain 01 and 02 at 37 °C were from Bacillus sp., Lactobacillus sp., Klebsiella sp., Azotobacter sp., and at 90 °C were from Lactobacillus sp., Klebsiella sp., Azotobacter sp., and Bacillus sp. The supernatant treated at 121 °C, with proteinase K and RNase A did not show the inhibitory effect. Furthermore, the supernatant from Pantoea sp. at pH 5, Bacillus sp. at pH 3, Klebsiella sp. at pH 3, Lactobacillus sp. at pH 8 and 10, and Klebsiella sp. at pH 10 showed the highest inhibitory effect against strain 01 and 02. The statistical analysis of mean difference was significant at the 0.05 level. The inhibitory activity of supernatant at pH 8-10 was reduced, comparing to supernatant at pH 5 which was the condition near the value of pH in bee stomach. Supernatant treated at 90 °C showed the higher inhibitory effect against strain 01 and 02, comparing to supernatant at 37 °C. In conclusion, gut bacteria which could inhibit P. larvae growth could be obtained from healthy bee.

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Student's Signature .....
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LIST OF ABBREVIATIONS

BHI agar	Brain heart infusion agar
bp	Base pair
°C	Degree Celsius
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
d-H_2O	Distilled water
EDTA	Ethylene diamine tetra-acetic acid
g	Gram
MIC	Minimal inhibitory concentration
TBE	Tris-boric-ethylene diamine tetraacetic acid
h	Hour
min	Minute
sec	Second
µl	Microlitre
µg	Microgram
mg	Milligram
16S rRNA	*16S ribosomal ribonucleic acid*
pol	*Poly protein*
RpS5	*Ribosomal protein S5*
ITS	*Internal transcribed spacer*
5.8S rRNA	*5.8S ribosomal ribonucleic acid*
mAU/ml	milli Anson unit
mM	Millimolar
ml	Millilitre
cm	Centimeter
mm	Millimeter
ppm	Part per million

%	Percentage
RT	Room temperature
rpm	Revolution per minute
v/v	Volume by volume
w/v	Weight by volume

CHAPTER I
INTRODUCTION

Cavity nesting honeybee, *Apis cerana,* is native to Thailand. It has provided pollination for many plants such as coconut, longan, rambutan and so on, which could have an economic impact to Thailand. There are many products produced by honeybee. For example, royal jelly is very popular for food because of nutrient enrichment. Beeswax has used as a natural ingredient for cosmetics and the main component of candles. Bee venom is well known for vaccine apitherapy, and honey has widely used for food [1]. In the view of honeybee's behavior, before it gets back to a nest after foraging, cleaning dance is always performed. Although other honeybees in the hive try to remove contaminated things including Varroa mite, microorganisms in nectar's flowers stored in forager's honey crop still remain [2]. Therefore, there are widely infections in an apiary caused by diseases or pests that have had impact on economic loss in apiculture for decades. Diseases in honeybee can be spread quickly since they live very closely in the hive. Therefore, any disease diagnostic technique at the early stage of infection is needed. Multiplex PCR is highly possible for this purpose.

For instance, American foulbrood disease (AFB) is caused by *Paenibacillus larvae*. AFB has an influence on the stages of larva and pupa. An infected larva turns brown and glue-like caramel. The spores of *P. larvae* can be found in *A. mellifera* hive and honey although no severe symptom can be observed by polymerase chain reaction (PCR). By this technique, generally, primers were designed from *16S rRNA* gene of *P. l. larvae* [3].

Moreover, nosema disease is caused by *Nosema ceranae* and *N. apis* which microsporidia are clustered in a group of fungi. A symptom is investigated by the swollen mid gut of honeybee. A light microscope can also be used to inspect *Nosema* spores but the limitation of this method is that the number of spores must be adequate. Moreover, this method is costly and time-consuming. Hence, in Canada, *Nosema* infection in *A. mellifera* was diagnosed and quantitated by the multiplex PCR

assay replaced the microscopy method. Where *16S rRNA* of *N. ceranae*, *N. apis*, and *RpS5* of *A. mellifera* were basically used to design primers [4].

Next, sacbrood disease is caused by sacbrood bee virus (SBV). This virus propagates in tissue which will block the synthesis of chitinase in larva. Eventually, this will affect the stunt pupation. Since the genetics of this virus is RNA, the one-step reverse transcription loop-mediated isothermal amplification primers sets (RT-LAMP) assay is used for sacbrood virus detection. The specific primers are designed based on *pol* of *SBV*. This method was simple and rapid because it inspected the change of color from orange to green [5].

Finally, chalkbrood disease is caused by *Ascosphaera apis* which can produces spores for reproduction. A larva (3-4 days old) is the most susceptible to this disease. An infected larva is swollen. After that, it will be wrapped by fiber like mummy. PCR assay was successfully performed to detect *A. apis* in *A. mellifera*. The specific primers were designed based on *internal transcribed spacer (ITS) 1-5.8S-ITS2* which were depended on species of *Ascosphaera* such as *A. aggregate*, *A. acerosa*, *A. atra*, *A. flava*, and *A. larvis* [6].

Moreover, Yoshiyama and Kimura reported the antagonistic effect against *P. larvae* from bacteria such as *Bacillus* sp. in the gut of *A. cerana japonica* [7]. Hence, the secondary metabolite or toxin from probiotics as microflora was healthful to living host in term of efficiency [8] in preventing or treating a disease [9]. Those bacteria were reported to be *Lactobacillus rhamnosus*, *L. acidophilus*, *L. reuteri*, *Bifaldobacterium infantis*, and *B. longum* [10]. In addition, Lactic Acid Bacteria (LAB) found in milk and preservatives could inhibit *P. larvae* [11].

In overall, the objectives of this research were

- to investigate infection of *P. larvae*, *N. ceranae*, *N. apis*, *SBV*, and *A. apis* in order to reveal the distribution of diseases in *Apis cerana* in Thailand,

- to analyse the partial DNA sequences of amplified PCR products in order to determine the similarity and phylogeny construction. As known, it could reveal the origin and antibiotic resistance of bee pathogens, and

- to identify gut bacteria in healthy *A. cerana* and determine the antagonistic effect of those gut bacteria against *P. larvae* in order to find an alternative way to protect and treat bee diseases. Later on, it would be useful in term of reducing antibiotic use in an apiary.

CHAPTER II
LITERATURE REVIEW

2.1 Biology of *Apis cerana*

About two million years ago from the Holocene and Pleistocene eras, it appeared that there were 6 species of *Apis* which were *A. cerana* [12], *A. dorsata* [12], *A. florea* [12], *A. andreniformis* [13], *A. mellifera* [14], and *A. koschevnikovi* [15]. In Asia, only *A. mellifera* (European honeybees) was imported from the Western countries, while the rest of 5 species were found in Asia [16]. In Thailand, there were 4 native honeybees which were *A. cerana* (cavity nesting honeybees), *A. dorsata* (giant honeybees), *A. florea*, and *A. andreniformis* (two dwarf honeybees).

The taxonomy of *A. cerana indica* was presented as below:
Taxonomy identification of *A. cerana indica* [17]

Kingdom Animalia

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Superfamily Apoidea

Family Apidae

Subfamily Apinae

Genus *Apis*

Species *Apis cerana*

Subspecies *Apis cerana indica*

In Southeast Asia, *A. cerana* could be classified into 8 subspecies. *A. cerana indica* [12] was distributed in Bangladesh, Burma, Indonesia, Malaysia, the Philippines, South India, Sri Lanka, and Thailand. *A. cerana cerana* [12] was distributed in Afghanistan, China, North India, North Vietnam, and Pakistan. *A. cerana japonica* [12] was distributed in Japan. *A. cerana johni* [18], *A. cerana heimifeng* [19], *A. cerana nuluensis* [15], and *A. cerana javana* [20] were distributed in Africa. In addition, *A. cerana skorikovi* [19] was distributed in Himalaya.

Considering the morphology, *A. cerana indica* looked like *A. mellifera* but the color and body size were different. An abdomen *A. cerana indica* was alternated dark black and yellow. In contrast, an abdomen *A. mellifera* was alternated dark yellow and black. For the body size, *A. cerana indica* was smaller than *A. mellifera* and *A. dorsata* but it was larger than *A. florea* and *A. andreniformis*. In addition, for behavior, *A. cerana* performed less aggressive behavior and less swarming than other 3 species which were *A. dorsata*, *A. florea*, and *A. andreniformis*. Furthermore, *A. cerana* habitat was hilly and flat areas. It hive was always found in a hole of timber (Figure 2.1). The average diameter of a nest was about 30 cm. It could adapt to subsist in any cavities similar to a hole of timber like a hive box (Figure 2.2) [21]. Hence, besides *A. mellifera*, *A. cerana* could be managed for apiculture [22].

Figure 2.1 Multiple-comb nest of *A. cerana indica* in a hole of timber from Samut-songkhram province. A photo was from Dr. Jirattikarn Kaewmaungmoon.

Figure 2.2 Multiple-comb nest of *A. cerana indica* in a hive box found in a rubber garden from an apiary in Chumphon province, Thailand.

A. cerana favored the winter full with plenty of flower nectar. Thus, it provided pollination for many plants such as coconut, longan, rambutan, berry, fodder plant, legume, medicinal plant, and so on, which could have an economic impact to Thailand. There are many products produced by this honeybee.

For example, royal jelly was very popular for food because of nutrient enrichment. Beeswax had used as a natural ingredient for cosmetics and the main component of candles. Bee venom was well known for vaccine apitherapy and honey had widely been used for food [1].

Behavior of honeybee was fascinating in general. The temperature of *A. cerana* nest was kept to be stable at 33-35 °C. In summer, they would perform fanning behavior by gathering outside of a nest and blowing wings in order to decrease the temperature inside the nest [23].

For behavior of reproductive swarming, *A. cerana* performed this behavior more often than *A. mellifera* when there was not sufficient nectar of flowers in surrounding area. [24].

A. cerana foraged food and water within an area of 1 km in diameter. After it returned to the nest, it would dance as a signal to communicate with other honeybees within the nest to know where the food source was [25].

In addition, before a forager got back to a nest after foraging, other bees inside the nest would perform cleaning dance in order to remove contaminated things including *Varroa destructor* mite (Figure 2.3), microorganisms in flower pollens. However, contaminated microorganisms in nectar stored in forager's honey crop still remained [2]. Therefore, there were wide infections in an apiary caused by diseases or pests that had had a large impact on economic loss in apiculture for decades [26].

Figure 2.3 *Varroa destructor* mite adhering to a larva's abdomen could be seen (http://en.wikipedia.org/wiki/List_of_diseases_of_the_honey_bee).

Diseases in honeybee nest can be spread quickly since bees live very closely in the hive. Therefore, any disease diagnostic technique at the early stage of infection is needed.

2.2 Diseases in bee

Diseases in bee were mostly caused by microorganisms such as bacteria, fungi, nematodes, virus, and protozoa [27]. In many countries, domestic animal department was responsible for controlling bee's pathogen. It was in charge of prescribing policy, examining, protecting, and assisting in an apiary in order to control and eliminate pathogens in the country [22].

For example, American foulbrood disease (AFB) was caused by *Paenibacillus larvae*. Nosema disease was caused by *Nosema ceranae* and *N. apis*. Chalkbrood disease was caused by *Ascosphaera apis*, and sacbrood disease was caused by sacbrood bee virus (SBV).

2.2.1 American foulbrood disease (AFB)

Paenibacillus larvae was the main cause for AFB. It had a strong influence on larvae and pupae. An infected larva turned brown and glue-like caramel (Figure 2.4 A). AFB had the prevalence over the world. Infected larvae would be dead within 10-15 days [28].

P. larvae was formerly called *Bacillus larvae*. In 1996, *16S rRNA* was used for classification and identification of bacteria. The *16S rRNA* sequence led to the new rearrangement of *B. larvae* into a new genus called *Paenibacillus*.

These bacteria produced spores in dormant stage. Inside spore, it contained genetic material which it could be able to grow in the unsuitable condition such as heat, cold, chemical. Hence, these bacteria were hard to eliminate. Haseman reported that pathogenic properties of spore had remained for 35 years [29]. Moreover, spores of *P. larvae* could germinate in the lumen of bee's mid gut within 24 h [27]. In Thailand, AFB was discovered since 1983 [17].

Spore would take about 10-15 days for hatching. A symptom of infected bee could be observed by the color change. An infected larva showed the color change from brown to black. In addition, the symptom could be observed in a field by using a matchstick [30]. When it was put into a suspected cell of a hive, the glue liked caramel of larva would be pulled out of the cell (Figure 2.4 B). Actually, this was the outstanding character of infected larvae. The identification in laboratory was possible because an infected larva was fluorescent. Also, the higher level of protease in infected bees could be assayed [31].

(A)

(B)

Figure 2.4 Morphology of AFB infected larvae. It becomes brownish (A), semi-fluid, and glue like caramel when it was pulled out of the cell gently by a matchstick (B)

(http://www.caes.uga.edu/departments/ent/bees/disorders/bacterial.html).

Susceptibility to *P. larvae* infection depended on age of larvae. The older larva got infected harder than the younger one [32]. Bamrick and Rothenbuhler found that a larva younger than 24 h could be sick by fewer than 10 spores of *P. larvae* whereas a larva older than 48 h could be sick by millions of spores [33]. It was explained that older bees had the thicker peritrophic membrane so *P. larvae* could not penetrate easily into their mid gut cell [34].

Susceptibility to *P. larvae* infection was not similar among castes which a queen was more susceptible than a worker and a drone [35]. In addition, bee species and strains affected the susceptibility. Danka and Villa found that Africanized honeybee was more resistant to AFB than *A. mellifera* [36]. For natural selection of honeybee, some strains could be able to resist AFB [37]. That might be related to the cleaning behavior of honeybee which could transfer infected larvae out of the hive and clean the contamination. This benefit led to the breeding of honeybee in order to produce the disease resistant strain.

Holst reported milk test for detecting the protease activity produced. Higher level of protease was produced by *P. larvae*. Briefly, a bee was laid down on a glass slide. Two drops of milk was transferred on the targeted bee. If the bee was sick by

AFB, the milk would be coagulated within 40 sec [38]. This result would not be observed if a bee was sick by European foulbrood or healthy. However, it was possible that delayed milk coagulation might be subsequently detected. Alternatively, using a microscope to investigate a spore of *P. larvae* in bee was also possible. Another method was to use a microbiological technique. A dead larva or bulk honey sample was cultured on agar media. The morphology of bacterial colonies such as color, shape, vegetative cell, could be seen. Also, a biochemical test or test kit such as BBL CRYSTAL was widely used [39]. Spores of AFB had been spread throughout the year although no external was found. It seemed to be that vegetative cell or rod shape cell did not cause a disease.

It was found that honey mixed with 10-hydroxydecenoic acid had the ability to inhibit *P. larvae* growth. However, if *P. larvae* already formed a spore, it could resist to 10-hydroxydecenoic acid. Thus, it could cause AFB infection to larvae [40]. Moreover, spore was found in fore gut of *A. mellifera* then to eliminating within mid gut. Interestingly, *A. mellifera* could release hydrogen cyanide gas to kill *P. larvae* better than other bee species [41]. Recently, AFB could be controlled by treating a hive with antibiotics like tetracycline. Nonetheless, left over antibiotics could be contaminated in bee products which led to the problem in exportation and consumption dramatically. If honeybees in an apiary were seriously infected, a beekeeper would burn the hive boxes. That led to the ruin in bee business [42]. Thus, developing a better technique to diagnose the spore contamination before the symptom revealed should be a better resolution. Recently, a molecular technique such as polymerase chain reaction (PCR) was applied for this purpose.

To detect the contamination of *P. larvae* spore in *A. mellifera* honey, PCR primers were designed from *16S rRNA* of *P. l. larvae* [3]. Furthermore, Antúnez et al. investigated the contamination of spores of *P. larvae* in honey collected from 19 provinces in Uruguay by PCR. The result presented that only honey from Colonia was contaminated by more than 500 colony forming unit (CFU) of *P. larvae* per gram of honey [43]. In contrast, honey from Cerro Largo, Rocha, Montevideo, Rivera, Artigas, and Salto were not contaminated.

2.2.2 Nosema disease

Nosema ceranae and *N. apis* were microsporidia in kingdom Fungi. They caused nosema disease which the symptom could be investigated by the swollen mid gut of honeybee. Nosema spores had the size about 2.5-5.0 μm. It grew within honeybee mid gut's epithelial cell. Moreover, nosema spores could grow in malpighian tubules and excretory organ [44]. Thus, this pathogen together with *Malpighamoeba mellificae* could work together to cause nosema disease. Furthermore, nosema spore could grow in hypo-pharyngeal gland [45]. It was believed that not only these microsporidia could cause nosema disease in bees, but it also caused the disease in other insects and invertebrates. Infected insects showed the symptom similarly to paralysis. Mid gut would be swollen with white and turbid color. The abdomen was unusual stretched and swollen. For invading mechanism, the parasites used the polar filament on germinating spore to pierce the host cells. Then, it replicated itself in cytoplasm and produced many spores.

Nosema could affect adult bees although they had strong exoskeleton. Considering a corpse of infected worker bee, it showed that spores could have been existed for 10 weeks to 4 months which depended on temperature. A queen died within 60 days after taking the spores [46]. The obvious symptom after infection was the digestive disorder, short life span, pollen storage reduction, queen supercedure induction, colony shrinkage, honey storage reduction, and high mortality (Figure 2.5). Nosema spores could be detected by microscopy. Many spores would be found from the grinded swollen gut under a microscope. Interestingly, pollen fed larvae had the higher infection rate than nectar fed larvae [40]. However, a microscope could not be used to detect low number of spores of nosema and it was time-consuming. Nosema disease had been spread worldwide. It caused the large scale losses in the US and the EU. The infection could be spread in many ways but the interesting one was from transportation of spores attached to the tools [47].

Figure 2.5 Nosema caused the digestive disorders, short bee life, pollen reduction, queen supercedure induction, decreasing colony, honey reduction, and colony mortality in the winter

(http://www.beekeepingsuccess.com/images/nosema.jpg).

An infected honeybee was necessarily separated from the others in order to prevent the spread of disease. Spores could exist up to 6 years in the frozen condition. On the other hand, it could be killed by heat at 57 °C for 10 min or at 21 °C within 60 days. The sunlight exposure could kill these spores within 15-51 h. For the treatment, the mixture of antibiotic like fumagillin B (0.1 g/ gallon of syrup) was very effective. In addition, an old hive box was sterile by incubated at 49 °C for 24 h. The infection could be controlled by removing an old comb and a hive box was treated by fumagillin.

In Canada, nosema infection in *A. mellifera* was diagnosed and quantitated by the multiplex PCR assay instead of the microscopy method. The *16S rRNA* of *N. ceranae*, *N. apis*, and *RpS5* of *A. mellifera* were basically used for primers [4].

Later, Whitaker et al. investigated *N. ceranae* and *N. apis* in 84 *A. mellifera* adults from 20 provinces in Turkey by multiplex PCR method. The result showed that number of bee infection by *N. ceranae* and *N. apis* was found to be 3 (3.57%) and 4 (4.76%) adults of *A. mellifera*, respectively [48]. However, 13 (15.48%) adults of *A. mellifera* were found not to get infected by both nosema species.

2.2.3 Chalkbrood disease

Chalkbrood disease was firstly found in 1968 [49]. It was caused by *Ascosphaera apis* which was in kingdom Fungi. The most outstanding character of infected larvae was a mummy like honeybee covered by fungi filament [50]. *A. apis* could produce many enzymes such as protease, β-N-acetyl glucosaminidase, alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase, and æ-mannosidase [51]. Theantana and Chantawannakul showed that protease and β-N-acetyl glucosaminidase had the major role for pathogen to penetrate the cuticle to the gut [52] of infected larvae [53]. Aronstein and Murray revealed that spores of *Aspergillus flavus* or *Aspergillus fumigatus* could germinate on larvae by using filament in order to penetrate into a lower cuticle layer [54].

Furthermore, spores of *A. apis* could germinate in alimentary tract and penetrate to cuticle layer of larvae within 2-3 days. The filament could produce a new spore on the outer layers of cuticle. At last, infected larvae would be swollen. After that, they would be wrapped in fiber like mummy which looked white due to the color of *A. apis* spore (Figure 2.6). Hence, the disease was called due to its appearance. Spores could exist longer than 15 years [55].

Figure 2.6 Chalkbrood infected larvae. It was wrapped in fiber like mummy (http://www.egofelix.com/7293-bee-culture-chalkbrood).

The spread of this pathogen could be from bee feeding equipment or spore contaminating soil. Epidemic and prevalence depended on environmental conditions such as temperature, moisture. Hence, chalkbrood disease was mostly found in warm climate areas [56].

There were many reports about the prevalence of *A. apis* in Europe [57], in Scandinavia, Russia, New Zealand [58], Argentina, Japan, the Philippines, Central America, and Mexico [59].

In Thailand, Wongsiri reported that the discovery of chalkbrood disease was not epidemic. At the beginning, it was found Chiang Mai province. Then, it was expanded to Pitsanulok province [17]. The affected colony had the rapid reduction of drones. Thus, it directly affected the breeding which led to the loss of honeybee population. Swarming was also noticeable. Nowadays, chalkbrood disease has been found in rainy season [60].

In term of using chemical reagents to eliminate the spore, there were many chemical reagents that effectively controlled chalkbrood disease such as sodium propionate, sorbic acid [61], citral, geraniol [62], and trichloro isocyanuric acid.

Gochnauer et al. found that treated infected honeybee with sorbic acid and sodium propionate mixed with pollen substitutes could reduce *A. apis* spores up to 50%. Moreover, that situation could make a queen return to be healthy [63].

Moreover, botanical compounds from plant extracts such as cinnamon oil, bay oil, clove oil, origanum oil, had been used to control *A. apis* and prevent chalkbrood disease. Calderone and Shimanuki discovered that the effective compound was azadirachtin, thymol, and **α**-terpinene. However, azadirachtin was easy to decompose in natural transformation of temperature and acid-base condition [64].

Besides, Maghrabi and Kish reported the relationship of Ascosphaerales causing bee diseases by using isozyme. Some species of *Ascosphaera* were successfully characterized [65]. Lu et al. used Random Amplified Polymorphic DNA (RAPD-PCR) to analyse those pathogens [55]. Later, Anderson and Gibbs constructed the phylogenetic tree of 20 species of *Ascosphaera* [66].

2.2.4 Sacbrood disease

Sacbrood bee virus (SBV) was the cause of sacbrood disease. In general, this virus would propagate in the inner cell membrane of larval tissue. It would block the synthesis of chitinase in larvae. Thus, pupation could not occur, comparing to a healthy larva which would become pupa within 4 days (Figure 2.7). An infected larva could induce the infection of other larvae even from other hives [67]. White founded that 1 infected larvae could ruin other 3,000 larvae per hive. Nevertheless, SBV could not severely spread in the nature because worker bees would find the infected larvae in the primary phase and removed it outside the hive [68]. Moreover, these viruses would lose its potential easily, especially after the larvae were dehydrated. In addition, SBV was well multiplied in a unique organ only which was hypo-pharyngeal gland locating in a head of worker bees and drones. Also, SBV disease was only founded in larvae stage of honeybee [69].

The color of infected larvae was changed from white into yellow and black brown, respectively, within 2-3 days. After the larvae died, it was dehydrated and became gondola-shaped [63].

Figure 2.7 Morphology of SBV infected larva. Pupation could not occur (http://agdev.anr.udel.edu/maarec/2011/02/07/summary-of-honey-bee-losses-for-de-and-md-2010/).

Sacbrood disease was spread in Europe, America, Austraria, and Thailand. Its genetic material was RNA. SBV had various strains either founded in *A. mellifera* L. or *A. cerana* [70].

In Thailand, SBV found in larvae of *A. cerana* was similar to what found in *A. mellifera* but the physical properties of both were different. Shah and Shah revealed that there was SBV infection in *A. cerana* at Doy Pui and Doy Aung-kang national parks. Bees from 2 hives were totally dead [71].

Protection and elimination of disease was performed by burning an infected hive and blanching relating equipment in boiling water.

Bailey et al. found SBV disease in adult honeybees (*A. mellifera*) in summer in Britain [41]. The diagnosis was by using one-step reverse transcription loop-mediated isothermal amplification primers sets (RT-LAMP) assay where specific primers were designed based on *pol* of SBV. This method was simple and rapid. It inspected the color change from orange to be green [5].

2.3 Phylogenetic analysis of bee pathogen

Aronstein et al. and Chen et al. revealed that three, and two distinct clades which comparing the sequences of high mobility group (HMG-box) genes [72], and small

subunit rRNA (SSUrRNA) genes of *A. apis*, and *Nosema* sp., respectively in *A. mellifera* [73].

Besides, Alippi et al. [74] and Grabensteiner et al. [75] reported that three distinct genotypes which analyzing the sequences of 16S rRNA genes (rDNA) of *P. l. larvae*, and five different regions of the SBV genome in *A. mellifera*.

Yoshiyama and Kimura reported the genetic diversity including the origin of *N. ceranae* by analyzing the partial sequence of *SSU rDNA* of *N. ceranae* in *A. mellifera* from Japan. They found two groups of *N. ceranae* from different geographic regions. Thus, it was assumed that the pathogen was contaminated to the imported honeybee products and a queen [76].

2.4 Antagonistic effect against *P. larvae*

In order to avoid the use of antibiotics in an apiary, alternative ways had been tried. There was plenty of research reporting the potential of isolated bacteria in bee body and bee products which could inhibit the growth of *P. larvae*. For example, Alippi and Reynaldi revealed 5 strains of *Bacillus* isolated from honey and apiaries of *A. mellifera*. They were *Bacillus megaterium* (m404), *B. cereus* (m6c), *B. cereus* (mv33), *B. cereus* (m387), and *B. licheniformis* (m347). Interestingly, those bacteria could inhibit the growth of 17 strains of *P. larvae* by *in vitro* inhibition assay [77].

Later, Forsgren et al. reported 11 lactic acid bacteria (LAB) isolated from honey of *A. mellifera*. They were phylotypes mixed in genus *Lactobacillus* and *Bifidobacterium* [78]. Also, they could inhibit the growth of *P. larvae* by *in vitro* inhibition assay. In addition, the larva feeding LAB mixture showed the significant decrease in number of infected colonies by *in vivo* inhibition assay. Besides, each of *Lactobacillus* phylotypes (Biut 2 and Hma 11) could inhibit the growth of 4 strains of *P. larvae* (ERIC I, II, III, and IV). *B. coryneforme* (Bma6) could inhibit the growth of 2 strains of *P. larvae* (ERIC III and IV) while it could partially inhibit the growth of 1 strain of *P. larvae* (ERIC II). In contrast, it could not inhibit the strain of ERIC I of *P. larvae*. Moreover, four genus of *Brevibacillus*, *Bacillus*, *Stenotrophomonas*, and *Acinetobacter* could inhibit the *P. larvae* growth by *in vitro* inhibition assay [79].

Reyes et al. revealed that 3 strains of *Flourensia tortuosa*, *F. riparia*, and *F. fiebrigii* isolated from plant extracts could inhibit the growth of *P. larvae* by *in vitro* inhibition assay [80]. Besides, essential oils from andiroba and copaiba could inhibit *P. larvae* growth as well [81].

Active metabolites from those potential bacteria were identified to be bacteriocin and pentocin. They were effective to many pathogens, both gram positive and gram negative bacteria, like *Salmonella* sp., but not fungi [82].

Those active metabolites, which were normally secreted out of the bacterial cells, were reported to be tolerant to harsh conditions. Ahmed et al. revealed that *L. acidophilus* supernatant remained the inhibition activity for about 25% after heat treatment at 121 °C for 20 min [83]. Moreover, the supernatant of *L. plantarum* (SH12 and SH24) isolated from butter made from camel milk showed the stable activity at pH 2 and 6 but lost the activity at pH 8. The supernatant of LAB isolated from gastrointestinal of fish, shellfish, and shrimp lost the activity at pH 6.5-7.0 [84].

For decades, American foulbrood (AFB) disease has been a severe disease so a beekeeper must burn hive boxes. That affects the economic loss in bee industry [33].

Hence, in this research, *A. cerana indica* which was economic and native to Thailand was focused. The distribution of bee pathogens was diagnosed and investigated by multiplex PCR (for pathogens with DNA as genetic material) and reverse transcriptase PCR (for pathogens with RNA as genetic material). Then, the phylogenetic relationship among those pathogens would be reported. Probiotic in the gut was identified by gram stain, fermentation, and *16S rRNA* analysis. Gut bacteria from healthy bee which could inhibit the growth of *P. larvae* were the interest.

CHAPTER III

MATERIALS AND METHODS

3.1 Equipments & Supplies

- Micro incubator, Taitec corporation, Japan
- PCR system 9700-96 well aluminium block, GeneAmp®, England
- Lamina flow, Renowm Technical Co., Ltd.
- Hybrilinker HL-2000, UVP®, Germany
- Benchtop UV Trans illuminator, UVP®, Germany
- Thermo spectronic, Thermo Scientific, UK
- Autoclave, Conbraco Industry Inc., USA
- Centrifuge/vortex combi-spin, Biosan, Latvia
- Refrigerate centrifuge, Sorvall®, Germany
- Non-refrigerate centrifuge, Eppendorf, USA
- Microplate reader, Titertek Multiskan® MCC/340
- Safety cabinet, Augusta, Thailand
- Microwave oven, Sharp Carousel R7456, Thailand
- Beaker, Pyrex®, Germany
- AA DISCS 2017-006, size 6 mm, Whatman International, Ltd., England
- Flask (50, 250, 500, and 1,000 ml in size), Schott Duran, Germany
- Microcentrifuge tube (1.5 ml in size), Sarstedt, Germany
- PCR® tubes, 0.2 ml clear thin wall, Genuine Axygen Quality, USA
- Plastic tube (15 and 50 ml in size), Sarstedt, Germany

- 96 well cell culture cluster, Costar®, cat. # 3599, USA
- Petridish, Sterilin, UK
- Automatic micropipettes (P10, P20, P100, P200, and P1,000), Gilson, France
- Anaerobic jar, Anaerocult®, Merck, Germany
- Pipette tips (200 and 1,000 µl in size), BioScience, Inc., USA
- Pipette tips (10 µl in size), Axygen Scientific, Inc., USA
- Electrophoresis chamber set, MGU-202T, CBS Scientific, USA
- Premiere® brand 9105 microscope slides
  (Single frosted glass, 75 x 25 x 1 mm^3 in size)

3.2 Chemicals

- API 50 CHB/E test, ref. # 50300 Biomerieux®, France
- API 20 A test, ref. # 20300 Biomerieux®, France
- Absolute ethanol (C_2H_6O, M = 46.07 g/mol), Merck KGaA Darstadt, Germany
- Emerald Amp GT PCR master mix, Takara, Japan
- AMV reverse transcriptase, Promega, USA
- 10 mM dNTP mix, Promega, USA
- QIAamp® DNA mini kit, cat. # 51304, Qiagen, USA
- QIAamp® RNeasy mini kit, cat. # 74104, Qiagen, USA
- QIAquick® PCR purification kit, cat. # 28104, Qiagen, USA
- Oxoid 2.5 L, AnaeroGen™, England
- Glycerin, Merck, Germany
- Agarose, low EEO, Research Organics, Inc., USA
- λ *Hin*dIII marker, Takara, Japan
- 100 bp DNA ladder marker, 5Prime, Germany
- Difco™ Brain Heart Infusion agar, ref. # 241830, USA
- Difco™ Brain Heart Infusion, ref. # 237500, USA
- Ethidium bromide ($C_{21}H_{20}BrN_3$, M.W. = 934.32), Bio Basic, Inc., USA

3.3 Sample collection

For disease diagnosis, ten apiaries were selected. The sampling period was in May and November, 2012. One natural apiary was in Samut-songkhram province and the rest were hive box apiaries in Chumphon province. In each apiary, five colonies were chosen. Within one colony, fifty of each larvae, pupae, and adults were randomly collected.

For gut bacteria, three colonies within the apiary in Samut-songkhram province were used. A criteria of collected healthy bee was that an apiary had to be in a natural condition. Bee population was dense. No dead bees surrounding a hive and the prevalent of disease were reported. Fifty healthy larvae and adults were collected.

All were preserved in dry ice and then, kept at -20 °C.

3.4 Disease diagnosis

3.4.1 Morphology

Larvae whether infected by American foulbrood disease (AFB) or not were determined by these characters like brownish body, semi-fluid, glue-like colloid. Additionally, bees whether attached with *Varroa destructor* mite or not observed by mites adhering to bee's abdomen as in Figure 3.1 [28].

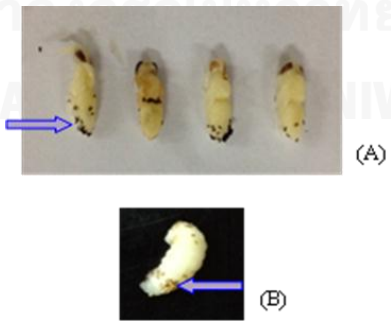

Figure 3.1 Varroa infecting larvae. Mites adhering to pupae's abdomen (A) and larva's abdomen (B) were indicated by arrows.

3.4.2 Multiplex PCR

DNA extraction

DNA of larva, pupa, and adults were extracted by DNA extraction mini kit (cat. # 51304, QIAgen). Bees (about 30 mg) were cut into small pieces by scissors and in 180 µl of ATL buffer. It was added by 20 µl of proteinase K and mixed by pipetting. Then, the mixture was incubated at 56 °C for overnight. The sample was quick spun in order to remove cell debris. Later, the cell lysate was added by 200 µl of AL buffer, vortexed, and incubated at 70 °C for 10 min. After it was mixed by 200 µl of 96-100% EtOH, it was spun at 6,000 rpm, RT for 1 min. Supernatant was transferred to a spin column. Then, it was spun at 8,000 rpm, RT for 1 min. Flow through (FT) was discarded. The spin column was washed by 500 µl of AW1 buffer. It was spun at 8,000 rpm, RT for 1 min. FT was discarded. The column was again washed by 500 µl of AW2 buffer. It was spun at 13,000 rpm, RT for 3 min. FT was again discarded. Then, the spin column was additionally spun at 13,000 rpm, RT for 1 min. After that, 30 µl of d-H_2O was added to the column in order to elute DNA. The soaked column was incubated at RT for 3 min. Next, it was spun at 6,000 rpm, RT for 1 min. The eluted DNA was kept at -20 °C until used. The concentration of DNA was calculated by the absorbance at 260 nm as the following formula in 3.1 and the purity of DNA was estimated from the formula in 3.2.

Concentration of DNA (µg/ml)

= (Abs 260) (50)* (dilution factor).....(3.1)

The purity of DNA

= Abs260**/Abs280***.....(3.2)

Definition: * Extinction coefficient of double-stranded DNA

** Absorbance at 260 nm

*** Absorbance at 280 nm

Another method to determine the quality of DNA is agarose gel electrophoresis.

RNA extraction

RNA was extracted by RNeasy mini kit (cat. # 74104, QIAgen). Samples (about 30 mg) were frozen in liquid N_2 and grinded in 600 µl of RLT buffer mixing with 6 µl of ß-mercaptoethanol. After that, the sample was spun at 13,000 rpm, RT for 3 min. The supernatant was mixed by 500 µl of 70% EtOH and transferred into a spin column. Later, it was spun at 10,000 rpm, RT for 15 sec. FT was discarded. The column was washed by 700 µl of RW1 buffer. It was spun at 10,000 rpm, RT for 15 sec. FT was discarded. The column was further washed by 500 µl of RPE buffer and was spun at 10,000 rpm, RT for 15 sec. After FT was discarded, the column was repeatedly washed by 500 µl of RPE. It was again spun at 10,000 rpm, RT for 15 sec. Finally, 35 µl of d-H_2O was added to the column. After it was incubated at RT for 3 min, it was spun at 6,000 rpm, RT for 1 min. Eluted RNA was kept at -20 °C until used. The concentration of RNA was measured by the absorbance at 260 nm using the formula in 3.3 while the purity of RNA was calculated from the formula in 3.4.

Concentration of RNA (µg/ml)

= (Abs 260) (40)**** (dilution factor).....(3.3)

The purity of RNA

= Abs260**/Abs280***.....(3.4)

Definition: **** Extinction coefficient of single-stranded RNA

** Absorbance at 260 nm

*** Absorbance at 280 nm

Another method to determine the quality of extracted RNA was formaldehyde/ agarose gel electrophoresis.

Agarose gel electrophoresis

Agarose [1.2% (w/v)] was prepared in 1x TBE buffer [for 10x TBE, 890 mM Tris base; 890 mM boric acid; and 20 mM EDTA (pH 8)]. A solidified gel was soaked in 1x TBE buffer as running buffer in a gel chamber. Five microlitres of PCR product was mixed with 1x loading dye [6x loading dye, 10 mM Tris-HCl (pH 7.6); 0.03% (w/v) bromophenol blue; 0.03% (w/v) xylene cyanol FF; 60 mM EDTA; and 60% (v/v) glycerol] and was loaded into a well. λ *Hin*dIII or 100 bp DNA ladder was used as DNA marker. Next, it was run at 80 volts for 45 min. After electrophoresis, it was stained by 10 μg/ml ethidium bromide (EtBr) for 5 min and was de-stained with d-H_2O for 20 min. Later, it was visualized under UV.

Formaldehyde/ agarose gel electrophoresis

A gel was prepared by mixing 1.2% (w/v) agarose with 100 ml of 1x MOPS buffer [10x MOPS buffer, 200 mM MOPS (pH 7.0); 80 mM sodium acetate; and 10 mM EDTA (pH 8.0)]. After melt and warm, 37% (w/v) formaldehyde was added into the mixture, mixed, and poured into a gel tray. After solidified, the gel was soaked in 1x MOPS containing 37% (w/v) formaldehyde at the ratio of 1: 5 as running buffer. After that, total RNA (0.5 μg) was mixed by 1x formaldehyde loading dye [5x loading dye (10 ml), 16 μl of saturated aqueous bromophenol blue solution; 80 μl of 500 mM EDTA (pH 8.0); 720 μl of 37% formaldehyde; 2 ml of glycerol; 3,084 μl of formamide; and 4 ml of 10x formaldehyde agarose gel buffer]. The sample was incubated at 65 °C for 3-5 min, cooled on ice, and loaded onto a formaldehyde/agarose gel. The electrophoresis was contained at 5–7 V/cm for at least 30 min. The gel was stained by 10 μg/ml of EtBr for 5 min and de-stained by d-H_2O for 20 min, respectively. Later, the electrophoresed RNA bands were observed under U.V.

3.4.3 Primer design

Total DNA was amplified by multiplex PCR. All designed primers were recorded as in Table 3.1. In order to detect the infection of AFB, in each reaction, it comprised of two pairs of primers which were based on Piccini et al. [3]. The first primer pair was designed according to the *16S rRNA* of *P. larvae* (accession # DQ 079621.1) which forward primer (F1) was PL5-F and reverse primer (R1) was PL4. The second primer pair was used as control which was designed according to *cytochrome b* of *A. cerana* (accession # ACR 55919.1) which forward primer (F2) was Cytb-F and reverse primer (R2) was Cytb-R.

In order to detect nosema, three pairs of primers were designed from *16S rRNA* of *N. ceranae* (accession # FJ 425736.1), of *N. apis* (accession # JX 860435.1), and ribosomal protein S5 (*RpS5*) of *A. mellifera* (accession # XM 393226.3) due to Hamiduzzaman et al. [4]. For *N. ceranae* detection, forward primer (F3) was MITOC-F while reverse primer (R3) was MITOC-R. For *N. apis* detection, forward primer (F4) was APIS-F while reverse primer (R4) was APIS-R. For *RpS5* as control, forward primer (F5) was RpS5-F while reverse primer (R5) was RpS5-R.

For detection of chalkbrood disease, two pairs of the specific primers designed which was depended on *5.8S rRNA* of *A. apis* (accession # HQ 905552.1) and *RpS5* of *A. mellifera* (accession # XM 393226.3) [6]. For *5.8S rRNA* amplification, forward primer (F8) was A. apis-F while reverse primer (R8) was A. apis-R. For *RpS5* amplification, forward primer (F5) was RpS5-F while reverse primer (R5) was RpS5-R.

For sacbrood virus (SBV) detection, two pairs of specific primers were designed which was based on polyprotein (*pol*) of SBV (accession # HG 779873.1) and *28S rRNA* of *A. mellifera* (accession # X 93388.1) [5]. For SBV detection, forward primer (F6) was SBV-F while reverse primer (R6) was SBV-R. For *28S rRNA* as control, forward primer (F7) was 28S-F while reverse primer (R7) was 28S-R.

Table 3.1 Designed primers for disease detection.

Primer name	Sequences (5'→ 3')	For detection of disease	Products size (bp)
PL5-F1	5'- TCAGT TATAG GCCAG AAAGC -3'	AFB	700
PL4-R1	5'- CGAGC GGACC TTGTG TTTCC -3'		
Cytb-F2	5'- TGATA AAAGA AATAT TTTGA -3'		500
Cytb-R2	5'- TGAAA CAAAT ATATA AATTG -3'		
MITOC-F3	5'- CGGCG ACGAT GTGAT ATGAA AATAT TAA -3'	Nosema	218
MITOC-R3	5'- CCCGG TCATT CTCAA ACAAA AAACC G -3'		
APIS-F4	5'- GGGGG CATGT CTTTG ACGTA CTATG TA -3'		321
APIS-R4	5'- GGGGG GCGTT TAAAA TGTGA AACAA CTATG -3'	Nosema	321
RpS5-F5	5'- AATTA TTTGG TCGCT GGAAT TG -3'	Nosema, Chalkbrood	115
RpS5-R5	5'- TAACG TCCAG CAGAA TGTGG TA -3'		
SBV-F6	5'- AAGGA ACTAT AGTAT GGCGA A -3'	SBV	200
SBV-R6	5'- CTGTT GCTGG TCTCT TGT -3'		

Primer name	Sequences (5' → 3')	For detection of disease	Products size (bp)
28S-F7	5'- AAAGA TCGAA TGGGG AGATT C -3'	SBV	358
28S-R7	5'- CACCA GGTCC GTGCC TCC -3'		
A.apis-F8	5'- TGTCT GTGCG GCTAG GTG -3'	Chalkbrood	500
A.apis-R8	5'- CCACT AGAAG TAAAT GATGG TTAGA -3'		

3.4.4 Polymerase Chain Reaction (PCR) amplification

A PCR reaction with final volume of 25 µl contained 12.5 µl of Emerald Amp GT PCR master mix (2x Premix, cat. # RR310Q, Takara), 5.0 µl of DNA template, 1.0 µl of 10 µM of each primer, and 0.5 µl of 10 mM deoxyribonucleotide triphosphate (dNTP mix).

For detection of AFB, the condition of PCR was set as followed. An initial denaturation step was at 94 °C for 1 min. It was followed by 35 cycles of denaturation step at 94 °C for 1 min, annealing step at 45 °C for 1 min, and extension step at 64 °C for 2 min. Then, the last extension was at 72 °C for 7 min.

Furthermore, for detection of nosema disease, the initial denaturation step was at 94 °C for 2.5 min. Then, it was followed by 10 cycles of denaturation step at 94 °C for 15 sec, annealing step at 55 °C for 30 sec, and the extension step at 72 °C for 45 sec. Continuously, it was followed by 20 cycles of denaturation step at 94 °C for 15 sec, annealing step at 50 °C for 30 sec, and extension step at 72 °C for 50 sec. Then, the last extension was at 72 °C for 7 min.

In addition, for detection of chalkbrood disease, the initial denaturation step was at 94 °C for 1 min. Then, it was followed by 30 cycles of denaturation step at 94 °C for 45 sec, annealing temperature at 45 °C for 1 min, and finally, extension step at 72 °C for 1 min. Then, the last extension was at 72 °C for 7 min.

After PCR amplification, all PCR products were observed by agarose gel electrophoresis.

3.4.5 Reverse Transcriptase PCR (RT-PCR) amplification

Since SBV has RNA as genetic materials, RT-PCR must be used instead of PCR. In each reaction, it contained final volume of 25 µl which composed of 12.5 µl of Emerald Amp GT PCR master mix (2x Premix), 5.0 µl of RNA template, 1.0 µl of 10 µM of each primer, 0.5 µl of 10 mM dNTP mix, and 0.5 µl of AMV Reverse Transcriptase (cat. # A1250, Promega).

At the beginning, the reaction cDNA was synthesized at 48 °C for 45 min. Then, it was amplified at 94 °C for 2 min and followed by 30 cycles of denaturation step at 93 °C for 1 min, annealing step at 50 °C for 30 sec, and extension step at 72 °C for 1 min. Then, the last extension was at 72 °C for 7 min. After the amplification was done, RT-PCR product was observed by agarose gel electrophoresis.

3.5 Phylogenetic tree of bee pathogens

3.5.1 PCR product purification

Targeted PCR product was purified by PCR product purification kit (cat. # 28104, Qiagen). Briefly, 1x volume of the PCR product was mixed by 5x volume of PBI buffer. Then, the sample was loaded into the Qiaquick column by pipetting and centrifuged at 10,000 rpm, RT for 1 min. After that, it was washed by 750 µl of PE buffer and centrifuged at 10,000 rpm, RT for 1 min. The flow through was discarded. Next, the column was transferred to a 2 ml collection tube and centrifuged at 10,000 rpm, RT for 1 min to remove the left over wash buffer. At last, the column was transferred to a 1.5 ml microcentrifuge tube and eluted by 30 µl of d-H_2O. After it was incubated at RT for 2 min, it was centrifuged at 6,000 rpm, RT for 1 min. The eluted PCR product was, then, sent for direct sequencing.

3.5.2 Direct sequencing

The purified PCR product was sent to AIT biotech (Singapore) for direct sequencing. After the nucleotide sequence was received, the similarity to the recorded sequences in GenBank database was searched.

3.5.3 Phylogenetic tree construction

The nucleotide sequences were analyzed by CLUSTAL X program. Next, the distance of relationships among the same pathogens was constructed by model distance test like neighbor-joining algorithms (NJ plot). A pairwise distance (P) model which the sequences were changed between each pair of taxa with bootstrap of 1,000 times was used.

3.6 Gut bacteria in healthy bee

3.6.1 Bacterial culture

External sterilization of larvae's body and healthy bees' gut was by dipping into 10 ml of 70% EtOH for 1 min, into 10 ml of 6% (w/v) sodium hypochlorite (NaOCl) for 1 min, and then, rinsed with 10 ml of sterilized water for 1 min. After that, either three larvae or three guts of healthy bees were homogenized in 700 µl of phosphate buffer saline (PBS). Next, the sample was spun at 6,000 rpm, RT for 1 min. Then, 200 µl of supernatant was spread on Brain Heart Infusion (BHI) agar (cat. # 241830, Difco). Two sets of experiments were done. Each set contained triplication. The first set was for aerobic bacteria. All plates were incubated at 37 °C for 18-24 h. The second set was for anaerobic bacteria. All plates were incubated in an anaerobic jar with a gas pak (cat. # AN 25US, Oxoid). They were incubated for 4-5 days [7].

3.6.2 Bacterial identification

Colony character

Colony morphology (color, luster, smooth, convex, or jagged edges) was observed and recorded. The number of colony was counted by colony forming unit using the formula in 3.5. The average in number was calculated from randomly 3 boxes of 1 cm^2 each.

Colony forming unit (CFU)/ ml =

$$\frac{[(\text{total colony from 3 boxes in } cm^2)*(\text{dilution factor})]}{\text{The volume used to spread plate (ml)}} \quad \ldots\ldots\ldots\ldots (3.5)$$

Definition: * $\frac{[\text{Area of plate } (\P r^2) \times \text{total colony in 3 } cm^2]}{\text{Area of boxes (3 } cm^2)}$

Gram staining

Briefly, one loop of colony was smeared onto a slide. The sample was dry by the flame. It was stained by 2 drops of crystal violet for 1 min and washed by 10 drops of d-H_2O. Later, it was soaked in 3 drops of iodine solution for 1 min and rinsed by d-H_2O. Again, the color was removed by 10 drops of 95% EtOH. This step was stopped by dipping into d-H_2O. After dry, the slide was stained by 3 drops of safranin O for 20-30 sec. At last, it was rinsed by d-H_2O and dry. The slide was viewed under a light microscope with oil [85].

Fermentation

It was observed by using API 20A test (cat. # 20300, Biomerieux) for anaerobic bacteria and API 50 CH test (cat. # 50300, Biomerieux) for aerobic bacteria. Briefly, one loop of the culture of targeted bacteria was inoculated into 100 ml of BHI medium (cat. # 237500, Difco) and incubated at 37 °C under the optimum condition. The opacity of culture was measured by the absorbance at 600 nm and was adjusted to be 0.5 McFarland [0.05 ml of 1.0% (w/v) barium chloride and 9.95 ml of 1.0% (v/v) sulfuric acid which presents 0.08-0.1 of absorbance at 600 nm and 74.3% transmittance with the interpretation of 1.5×10^8 CFU/ml]. After that, 100 µl of the targeted bacterial culture was spread onto BHI agar. All colonies from one plate were swept by cotton bud and transferred into an ampoule containing 5 ml of d-H_2O. Then, X drops were transferred into other ampoule containing 5 ml of 0.85% NaCl until the turbidity reached the McFarland standard point of 2 for API 50 CHB/E medium and 3 for API 20 A medium, respectively. The number of X drops was counted and recorded. Next, 2X drops from the ampoule containing 5 ml of d-H_2O with colonies was added into an ampoule containing 10 ml of medium and mixed by pipetting. Then, 200 µl of the mixed medium was transferred onto each of blank strips. All strips were incubated at 37 °C for 18-24 h for API 50 CHB/E test and incubated at 37 °C for 2-3 days for API 20 A test. At last, the changed color on strips was observed. For the positive result, the red would be changed to be yellow for API 50 CHB/E and the purple would be changed to be yellow for API 20 A test, respectively. The results were interpreted by searching the data in the website of “www.apiweb.bio.merieux.com” with the username of “SSNP” and the password of “SNP8179031”.

Sequence analysis of 16S rRNA gene

By doing colony PCR, 1 colony was picked into 20 µl of 10 mM TE buffer (pH 7.5). It was kept at -20 °C for 30 min. After that, it was incubated at 96 °C for 5 min and vortexed. This step was repeated 3x. Next, it was amplified by using forward primer (eu27F) which was 5'- GAGAG TTTGA TGCTG GCTCA G -3' and reverse primer (eu1495R) which was 5'- CTACG GCTAC CTTGT TACGA -3' [7]. PCR reaction was performed in a final volume of 25 µl containing 12.5 µl of Emerald Amp GT PCR master mix (2x Premix). Then, 8 µl of nuclease free d-H_2O and 2 µl of DNA templates were added. Next, 1 µl of 10 µM of each primer and 0.5 µl of 10 mM dNTP mix were transferred. The condition of PCR was initially started with the denaturation step at 95 °C for 1 min. It was followed by 35 cycles of denaturation step at 95 °C for 1 min, annealing step at 55 °C for 1 min, and extension step at 72 °C for 1 min. Then, the last final extension step was at 72 °C for 7 min. After PCR, the amplified product was checked by 1.2% agarose gel electrophoresis and EtBr staining or gel red. The expected size of PCR product was about 1.4 kb.

3.7 Antagonistic effect of gut bacteria on AFB

Agar well diffusion assay was used for this purpose. Two strains of *Paenibacillus larvae*, strain 01 and strain 02, were chosen. In brief, one loop of strain 01 or strain 02 glycerol stocks was separately cultured in 100 ml of BHI medium and incubated aerobically at 37 °C for 3 days. The opacity of culture was measured by the absorbance at 600 nm and was adjusted to be 0.5 McFarland. After that, 100 µl of strain 01 or strain 02 culture was spread onto BHI agar. A small well at the center of BHI agar plate was drilled by using 1,000 µl of pipette tip (diameter of tip was 7 mm).

Targeted gut bacteria were suspended in d-H_2O and was adjusted to be 10^6 cells/ml. After that, 100 µl of gut bacterial culture was dropped into a well of an agar plate. It was incubated at 37 °C for 18-24 h in aerobic condition for aerobic gut bacteria. On the other hand, plates were incubated at 37 °C for 4-5 days in anaerobic condition by using an anaerobic jar with gas pack for anaerobic gut bacteria. Later, the diameters of inhibition zone were measured. The experiment was performed in triplication. BHI

medium was used as negative control. The result was reported in term of mean and standard deviation.

3.8 Antibiotic resistance of *P. larvae*

Disc diffusion method was used for this purpose. One colony of strain 01 or strain 02 strains were added into 100 ml of BHI medium separately and were incubated at 37 °C for 3 days. Then, 100 µl of culture was spread onto BHI agar plate. Selected antibiotics were nalidixic acid, streptomycin, tetracycline, penicillin, and ampicillin. An antibiotic at various concentrations was prepared by serial dilution with d-H_2O. All plates were incubated aerobically at 37 °C for 3 days. The diameter of inhibition zone around the discs (cm) was measured. The experiment was performed in triplication. BHI medium was used as negative control. The result was reported in term of mean and standard deviation.

3.9 Antibacterial activity of gut bacteria's supernatant

One colony of targeted bacteria was inoculated into 25 ml of BHI medium. It was incubated at 37 °C for 1 day under both aerobic and anaerobic conditions. After that, 500 µl of the culture was transferred and spun at 9,000 rpm, RT for 15 min. One hundred microlitres of cell free supernatant was mixed with 100 µl of strain 01 or strain 02 cultures (10^4 CFU/ml). Then, it was transferred into a well of 96 well plate. Additionally, 100 µl of strain 01 and strain 02 culture without the cell free supernatant was used as positive control while 100 µl of cell free supernatant mixing with 100 µl of BHI medium was used as negative control. All samples were incubated aerobically at 37 °C for 1, 2, and 3 days. Bacterial growth was calculated from the measured O.D. 560 by using micro plate reader. The calculation was based on mean value of O.D. 560 from three replications. The percentage of growth of strain 01 and strain 02 was estimated according to the following formula [86].

$$\% \text{ of growth} = \frac{\text{O.D. 560 after 1, 2, or 3 day incubation} - \text{O.D. 560 at 0 h}}{\text{O.D. 560 at 0 h}} \times 100$$

3.10 Effect of heat treatment on supernatant to antibacterial activity

Cell free supernatant was heat treated at 90 °C and 121 °C for 1 h. One hundred microliters of heated cell free supernatant was mixed with 100 µl of strain 01 or strain 02 culture in a well of 96 well plate. Next, it was incubated aerobically at 37 °C for 1, 2, and 3 days. Triplication in experiments was done. The percentage of growth of strain 01 or strain 02 was calculated as previously mentioned [87].

3.11 Effect of enzymes on supernatant to antibacterial activity

One microliter of > 600 mAU/ml of proteinase K (cat. # 19131, Qiagen) or 100 mg/ml of RNase A (cat. # R6513, Sigma) was mixed with 1 ml of cell free supernatant and incubated at RT for 1 h. After that, 100 µl of treated cell free supernatant was mixed with 100 µl of strain 01 or strain 02 culture and was transferred into a well of 96 well plate. Next, it was incubated aerobically at 37 °C for 1, 2, and 3 days. Triplication in experiments was done. The percentage of growth of strain 01 or strain 02 was calculated as previously mentioned.

3.12 Effect of pH on cell free supernatant to antibacterial activity

The value of pH on cell free supernatant was adjusted to be 3, 5, and 6 by using 0.1 N HCl and 0.2 N NaOH. Then, 100 µl of treated cell free supernatant was mixed with 100 µl of strain 01 or strain 02 culture and was transferred into a well of 96 well plate. Next, it was incubated aerobically at 37 °C for 1, 2, and 3 days. Triplication in experiments was done. The percentage of growth of strain 01 or strain 02 was calculated as previously mentioned.

3.13 Statistical analysis

The data was analyzed by a program of SPSS statistics, version 17.0. The confidence at 95% was determined. The p-value less than 0.05 would indicate the significant difference. Unpaired t-test was used for two independent samples while one way ANOVA and post hoc test by Fisher's least significant difference (LSD) were used for more than two samples.

CHAPTER IV
RESULTS

4.1 Sample collection

For disease diagnosis, 50 colonies were randomly collected from Muang district, Samut-songkhram province (5 colonies), and Muang district (5 colonies), Lang Suan district (15 colonies), Lamae district (20 colonies), Sawi district (5 colonies), Chumphon province in May 1, November 19-21, 2012 and March 10, 2013, respectively. The coordinates were at 13° 24’ N, 100° 01’ E, at 10° 29’ N, 99° 10’ E, at 11° 20’ N, 99° 29’ E, at 9° 46’ N, 98° 56’ E, and at 10° 15’ N, 99° 54’ E, respectively.

For gut bacteria, 3 colonies within the apiary in Samut-songkhram province were collected in July 2, 2012 with the coordinate of 13° 24’ N, 100° 01’ E as showed in Table 4.1.

Table 4.1 Sampling sites for bee collection.

Date of sample collection	**Sampling site**	**Number of apiaries (5 colonies/ apiary)**	**Coordinates**
May 1, 2012	Muang district, Samut-songkhram	1	13° 24’ N, 100° 01’ E
July 2, 2012 (Healthy bees)	Muang district, Samut-songkhram	1 (3 colonies/ apiary)	13° 24’ N, 100° 01’ E
November 19-21, 2012	Muang district, Chumphon	1	10° 29’ N, 99° 10’ E
	Lang Suan district, Chumphon	3	11° 20’ N, 99° 29’ E, 10° 2’ N, 99° 3’ E, and 10° 1’ N, 99° 3’ E
	Lamae district, Chumphon	3	9° 47’ N, 98° 56’ E
March 10-12, 2013	Lamae district, Chumphon	1	9° 46’ N, 98° 56’ E
	Sawi district, Chumphon	1	10° 15’ N, 99° 54’ E

4.2 Disease diagnosis

4.2.1 Morphology

The morphology of randomly sampled bees from 50 colonies was observed and reported in Table 4.2. Bees at larva, pupa, and adult stages were observed. Considering larvae, some larvae from 6 colonies (15 samples) were attached by mites. Some larvae from 8 colonies (75 samples) looked black and brown. Considering pupae, some pupae from 16 colonies (74 samples) were attached by mites. Some pupae from 2 colonies (9 samples) looked black and brown. In contrast, no adult was attached by mites and infected.

4.2.2 Multiplex PCR

DNA extraction

The quality of extracted DNA from larvae, pupae, and adults of *A. cerana* was assayed by 1.2% (w/v) agarose gel electrophoresis. After EtBr staining, the band of DNA with good quality was sharp with high M.W. (Figure 4.1A). Approximately, it was about 23 kbp.

RNA extraction

The quality of extracted RNA from larvae, pupae, and adults of *A. cerana* was assayed by 1.2% (w/v) formaldehyde/ agarose gel electrophoresis. After EtBr staining, the sharp bands of *18S* and *28S rRNA* were appeared (Figure 4.1B). Furthermore, the smear of mRNA at the medium to high M.W. could be observed.

Table 4.2 Diagnosis of bees by morphology observation.

Apiary #. colony #	Number of infected bees			
	Mites (*Varroa* spp.)		Black and brown color	
	Larva	Pupa	Larva	Pupa
2.1	3	0	0	0
2.3	4	0	0	0
3.1	0	10	27	0
3.2	0	12	0	0
3.3	0	1	1	0
3.4	0	0	3	0
3.5	0	0	4	0
4.1	0	0	3	0
4.2	0	3	4	0
4.4	0	1	0	0
4.5	0	1	0	0
5.2	1	0	0	0
5.3	0	8	0	0
5.5	0	5	27	6
6.3	0	3	0	0
6.4	0	2	0	0
6.5	0	5	0	0

Apiary #. colony #	Number of infected bees			
	Mites (*Varroa* spp.)		Black and brown color	
	Larva	Pupa	Larva	Pupa
7.1	0	3	0	0
7.3	1	13	0	3
7.4	1	3	0	0
8.1	0	0	1	0
8.5	0	3	0	0
10.2	0	1	0	0
10.5	5	0	0	0
Total	15	74	75	9

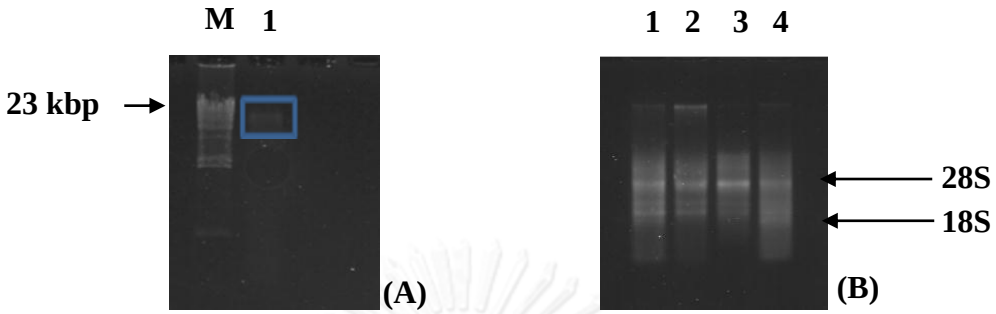

Figure 4.1 DNA and total RNA extracted from larvae, pupae, and adults of *A. cerana*. For 4.1A, lane M was λ *Hin*dIII marker and lane 1 was extracted DNA. For 4.2B, lanes 1-4 were extracted RNA.

4.2.3 Polymerase Chain Reaction (PCR) amplification

After diagnosing by multiplex PCR, detected *P. larvae* (4.2A), *A. apis* (4.2B), *N. ceranae* and *N. apis* (4.2C), and *pol* (4.2D) were shown in Figure 4.2. The percentage of infected bees could be estimated (Table 4.3). Collected bees from Samut-songkhram province were infected by *P. larvae*, *N. ceranae*, and *N. apis* at 2 (4%), 3 (6%), and 1 (2%) sample(s), respectively. Also, 8 (16%), 1 (2%), and 10 (20%) of collected bees from Chumphon province were infected by *P. larvae*, *N. apis*, and *A. apis*, respectively. For both provinces, it showed that 20%, 6%, 4%, and 20% of samples were infected by *P. larvae*, *N. ceranae*, *N. apis*, and *A. apis*, respectively. Moreover, bees those were not infected by *P. larvae*, *N. ceranae*, *N. apis*, and *A. apis* were at 80%, 94%, 96%, and 80%, respectively.

Considering the above data and Table 4.3, it seemed to be that bee stage was also important for infection. In overall, from total of 50 colonies, the number of *P. larvae* infected bees classifying in stage development was 8 from larva, 9 from pupa, and 8 from adult bees. The number of *N. ceranae* infected bees classifying in stage development was 2 from larva and 3 from adult bees. The number of *N. apis* infected bees classifying in stage development was 3 from adult bees only. In addition, the number of *A. apis* infected bees classifying in stage development was 8 from larva, 8 from pupa, and 3 from adult bees.

In this research, the frequencies of infection by *N. ceranae* and *N. apis* were lower in larvae comparing to adults. Also, infection by *A. apis* was more prevalent in larvae and pupae than in adults while the most prevalence of infection by *P. larvae* was in pupae.

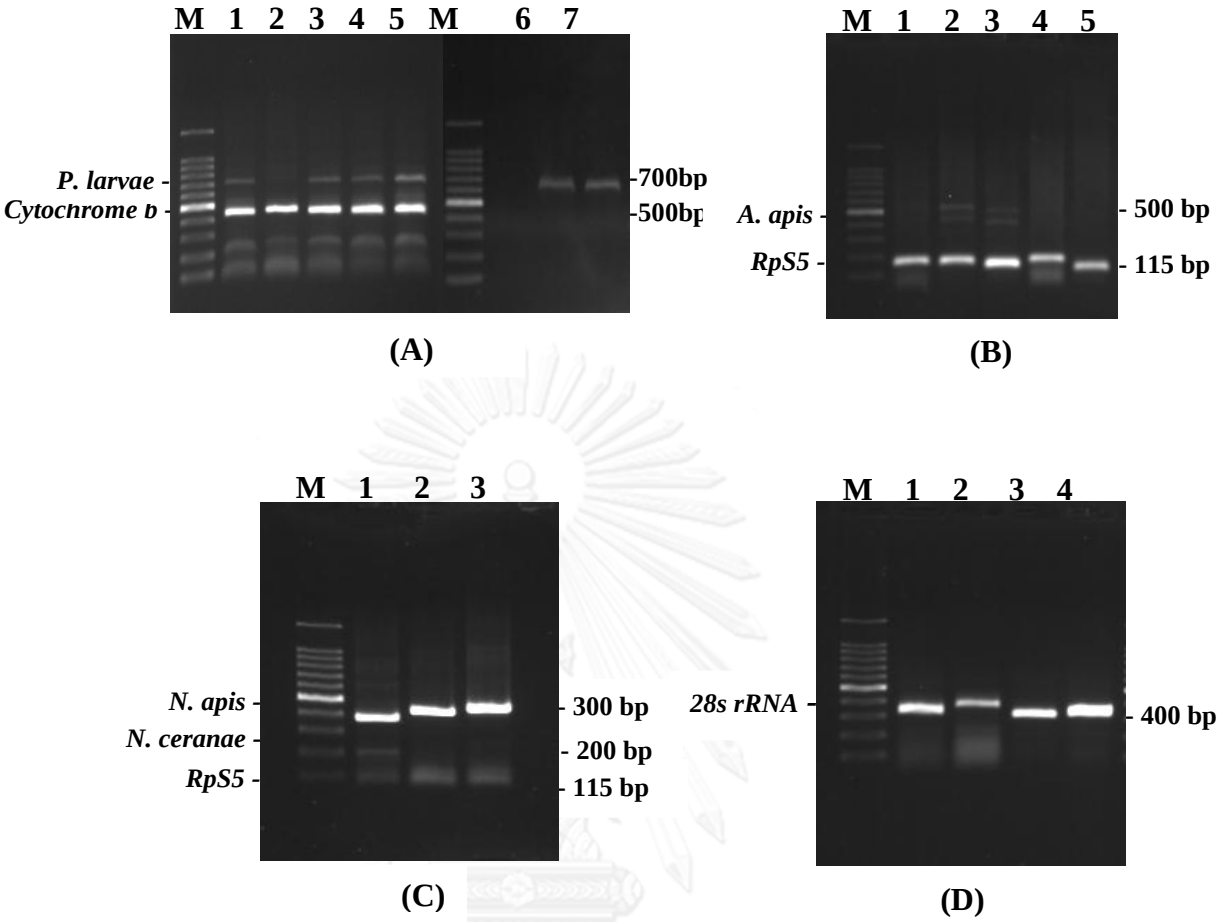

Figure 4.2 Multiplex PCR presenting *P. larvae* (A), *A. apis* (B), *N. ceranae* and *N. apis* (C), and *pol* (D) infection. Lane M in all figures was 100 bp DNA ladder. For 4.2(A), lanes 1-5 contained *cytochrome b* amplified products (500 bp) as control and lanes 1, 3-5 contained *P. larvae* amplified products (700 bp). Also, lanes 6-7 contained spores of *P. larvae* amplified products (700 bp) as positive control. For 4.2(B), lanes 1-5 contained *RpS5* amplified products (115 bp) and lanes 2-3 contained *A. apis* amplified products (500 bp). For 4.2(C), lanes 1-3 contained *RpS5* amplified products and lanes 1-3 contained *N. apis* amplified products (300 bp). Besides, lane 1 contained *N. ceranae* amplified products (200 bp). For 4.2(D), lanes 1-4 contained *28S rRNA* amplified products (400 bp) as control but not *pol* amplified products (200 bp) at all.

Table 4.3 Infection of *P. larvae*, *N. ceranae, N. apis,* and *A. apis* in *A. cerana* in Thailand.

Province	Number of collected colonies	Number of colonies infected by			
		P. larvae (Bee stage)	*N. ceranae* (Bee stage)	*N. apis* (Bee stage)	*A. apis* (Bee stage)
Samut-songkhram	5	2 (pupa 1, adult 2)	3 (larva 2, adult 3)	1 (adult 1)	0
Chumphon	45	8 (larva 8, pupa 8, adult 6)	0	1 (adult 1)	10 (larva 8, pupa 8, adult 3)
Total	50	10 (20%)	3 (6%)	2 (4%)	10 (20%)

4.3 A phylogenetic tree of bee pathogens

4.3.1 Partial DNA sequence

The partial sequences of *16S rRNA* gene of *P. larvae* from 10 samples (Figure 4.3), of *N. ceranae* from 3 samples (Figure 4.4), of *N. apis* from 2 samples (Figure 4.5), and of *5.8S rRNA* gene of *A. apis* from 10 samples (Figure 4.6) were analysed and searched for the similarity to the recorded sequences in GenBank database. The percentage of maximal identity of *P. larvae*, *N. ceranae*, *N. apis*, and *A. apis* was shown in Table 4.4.

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....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70         80
AAGCGATCAA ATATTCTCGA ATCCTCGAAT TCAACCAACT TCGATCAATC GACAAGTTCA ATCGTACAAC GAATCGACGA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         90        100        110        120        130        140        150        160
CACCAAAATA CGGCATCAAA TTTGCGAGTA AGAACATGAG ATATCTTAAC CTAGACGGAA GCGTGGAGCC CGCCATTAAG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        170        180        190        200        210        220        230        240
AAGAACGGCG ATGGCGGGGA ATTCAAATCA ACAGAAATGG ATCCCGTCGT CTCGAAAGTG CCTAAACCCG TCGATAGCCA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        250        260        270        280        290        300        310        320
TTGGAGAGTG CTTCGAGGCG TAAATCTACG ATCGAAAGAG GCAATAGTGT CGGCGAATAA ACAGAGTCAT GGAGGTTCTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        330        340        350        360        370        380        390        400
GAGAAAGAAG CGATCGCAAC GATGATGCGT TTTTTAGAGG CGTCCCGGAG GTCTAATGAT AGCGCGATGA ATTATTCACG

....|....| ....|....| ....|....| ....|....| ...
        410        420        430        440
AGGAAACACA AGGTCCGCTC GAATCACTAG TGAATTCGCG GCC
```

Figure 4.3 The partial sequence of *16S rRNA* gene from *P. larv*ae.

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70         80
CTAATGGAGA TTATAGCATT TGAGTTTTTT GGCTCTGGGG ATAGTATGAT CGCAAGATTG AAAATTAAAG AAATTGACGG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         90        100        110        120        130        140        150        160
AAGAATACCA CAAGGAGTGG ATTGTGCGGC TTAATTTGAC TCAACGCGAG GTAACTTACC AATATTTTAT TATTTTGAGA

....|....| ....|....| ....|....|
        170        180        190
GAACGGTTTT TTGTTTGAGA ATGACCGGGA
```

Figure 4.4 The partial sequence of *16S rRNA* gene from *N. ceranae*.


```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70         80
CCCCAAAGCG TGCTTTTCAT AAGTGGTGTG CGATATTGAT AGCTTGGGAG AGTGGTAACC CTTGGTGAAA GGGGACCTTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         90        100        110        120        130        140        150        160
TATAAACCTG AGAAGCACGT CGGTCTCTCG GGGTTTTCTC ATTCCGCAGT TGCCCCGTTC TCCGGTGTCC ACTCAATTGT

....|....| ....|....| ....|....| ....|....| ....|.
        170        180        190        200
GGGAGGTCAA TTATTAAACT CGGCCGATGA TCTTTAAGAT GGTAGC
```

Figure 4.5 The partial sequence of *16S rRNA* gene from *N. apis*.

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|

         10         20         30         40         50         60         70         80
GGGGTTGGGG GAACGGGGGA AACCCGACGA ACGGGCACGA CTACTCATGG GAAAAAAAAC GTAACCGGCG GGGGGGAGAT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          90        100        110        120        130        140        150        160
CCTGGCCACC TATACCTCAC AGTCATGGTT TCTCTTCGGA TCTTTCTCTC GCACCTCGTC CGCACTAGAC AGGTTATGAC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         170        180        190        200        210        220        230        240
TTAATTCGTC TTTTTTTGAA GCAAAAAAAG ATTATTATTG CGCTTATTCT GAACAATTTT TTCCTTTTTG CATTCTTATT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         250        260        270        280        290        300        310        320
GTTTTAAATA TGCGCTGCCC CATATCGCTT CTTGTCTTAT TCATATTCAT GCCCATGCTC TCGCGATTGT TTTGTTTTAA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         330        340        350        360        370        380        390        400
CGCCCGTCCT GTATTTTAGC TTCCGTTGAC AATTCCCCCG ACCAAATAAT TAATTCTGCT TTATGTTAAG CGTTATGCCT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         410        420        430        440        450        460        470        480
GGTTGGCCGT ATGCAACCCT TTTTCTTCTC TTTTTTTTAA TTATTTCTTT CTTTTCGACT TTTCGAAGGT CCCCGTTTTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         490        500        510        520        530        540        550        560
ATTTCTTTTT TTTCCAACGA CCTCTTTATT TTTCTTGAGT GTATGGGGGT GGTTCTTCGT TCTAGTTGTT TTTCTATTCT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         570        580        590        600        610        620        630        640
CTTTCTTTTT TATCATTTAT TTCTTGTGGT TGACTTTGCG ATTATTTATG GTTCCTCGCT CTACTTGCTG ATATTATACG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         650        660        670        680        690        700        710        720
TTGCATGTTC GCCGATGCCA AAGTGAGCGG GTTCTTTGCT GAAGAAACGG ACCATAAAAC CGTCATCACA CTGCTTTCAC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         730        740        750        760        770        780        790        800
AAGCAGATAA TATCCCAGAT TGCCCTTACG CTCTACGACT CGTTCTAACT CAACTGGCGT GCAACCTGTT CTCCTCACCC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         810        820        830        840        850        860        870        880
GTCTACCCTG ATAAAATTTC ATCGTCGCCT GTTTTGAAAG AGACATGCAT CAAGCTAGCG ACCTCTTCGA TGTTGGACTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         890        900        910        920        930        940        950        960
GCATGCTACA TTACGAACCG CAGCTGTCTC ACTGATATAC AACTTGGCAT CCTTTAATCA TAATGCTCGG CTCGACGAGC

....|....| ....|....| ....|...
         970        980
GGCCGGAAAA GCTTTCAGAG ATAGAACA
```


Figure 4.6 The partial sequence of *5.8S rRNA* gene from *A. apis*.

Table 4.4 The percentage of maximal identity of bee pathogen sequences.

Bee pathogen	Accession #	Description	Length of identity
Paenibacillus larvae	AY030079.1	*Paenibacillus larvae* subsp. *larvae* *16Sribosomal RNA* gene, partial sequence	94-100%
Nosema ceranae	KC708007.1	*Nosema ceranae* strain N5J small subunit *ribosomal RNA* gene, partial sequence	98-99%
Nosema apis	U76706.1	*Nosema apis 16S ribosomal RNA* gene, partial sequence	95-100%
Ascosphaera apis	HQ905552.1	*Ascosphaera apis* isolate KVL08-041 marker Sca1635 genomic sequence	95-100%

4.3.2 Phylogenetic tree construction

The sequences of amplified gene were also used to construct phylogenetic trees by neighbor-joining algorithm (NJ plot). The phylogenetic lineage depended on the partial sequences of *16S rRNA* gene of *P. larvae*, *N. ceranae*, *N. apis*, and *5.8S rRNA* gene of *A. apis* were exhibited six, two, single, and four distinct clades as showed in Figures 4.7, 4.8, 4.9, and 4.10, respectively.

Interestingly, a phylogenetic tree of *16S rRNA* gene in *P. larvae* revealed that the obtained sequences from both Samut-songkhram and Chumphon provinces were closely related to the recorded *Paenibacillus* genus. Due to Figure 4.7, it exhibited 6 distinct clades and could be grouped into 6 clusters (Cluster I, II, III, IV, V, and VI) which were very closely related in phylogenetic lineage. However, the sequence from Chumphon province was not in a monophyletic group because the sequence from Samut-songkhram province was inserted between the sequences from Chumphon province. Furthermore, it showed that the different geographic regions in Samut-songkhram and Chumphon provinces did not affect to transfer the genetics of *P. larvae.* A *16S rRNA* gene sequence of *P. montaniterrae* was used as an out-group with 1,000 bootstrap replications. However, the sequences of *P. larvae* in cluster I, II, III, IV, V, and VI were found in different areas where dominant plant species were palm, rambutan, longan, pineapple, coconut, and rubber trees, respectively. Therefore, *P. larvae* in cluster I, II, III, IV, V, and VI shared the common ancestor more than one as a paraphyletic group.

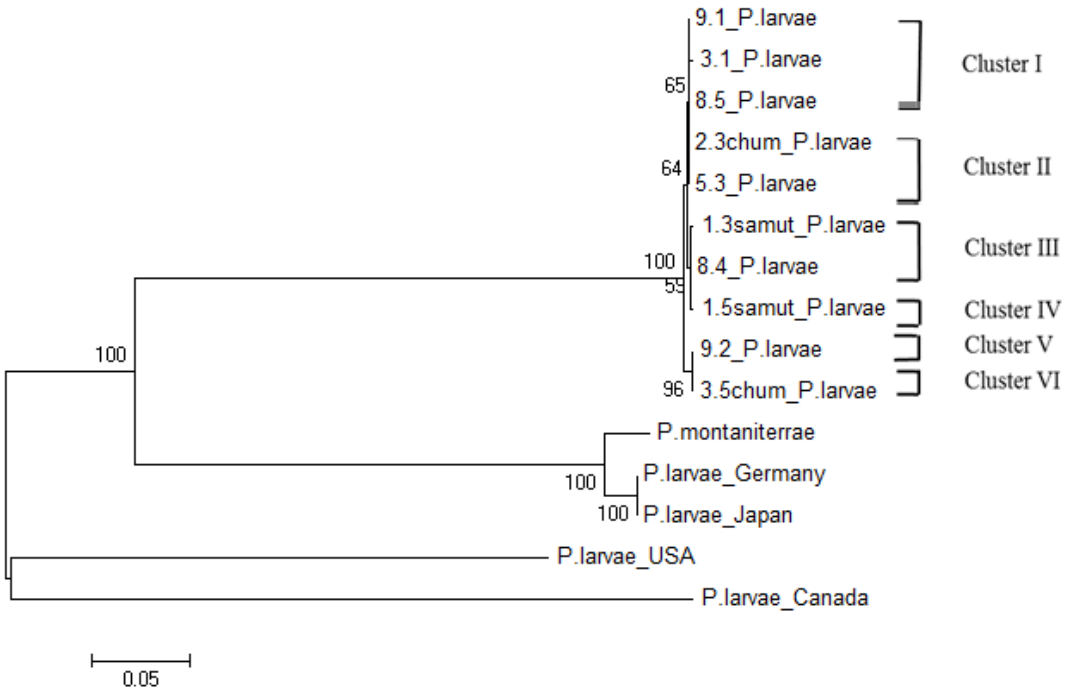

Figure 4.7 An NJ tree of the aligned nucleotide data set of *16S rRNA* gene sequences from *P. larvae* (700 bp). Ten isolates were from Samut-songkhram and Chumphon provinces while the rest were from other countries. A *16S rRNA* gene sequence of *P. montaniterrae* was used as an outgroup for the analysis. The number under branches indicates the percentage of bootstrap supported with 1,000 replications.

Considering a phylogenetic tree of *16S rRNA* gene in *N. ceranae*, it revealed that a sequence from Samut-songkhram province was closely related to a sequence of *Nosema* genus. It exhibited two distinct clades (Cluster I and II) as in Figure 4.8. Cluster I contained sequences of *N. ceranae* found in longan and santol trees. In contrast, cluster II contained a sequence of *N. ceranae* found in mimosa and coconut trees. A sequence of *16S rRNA* gene in *N. bombycis* was used as an out-group with 1,000 bootstrap replications. Therefore, *N. ceranae* in cluster I and II showed common ancestor more than one as a paraphyletic group.

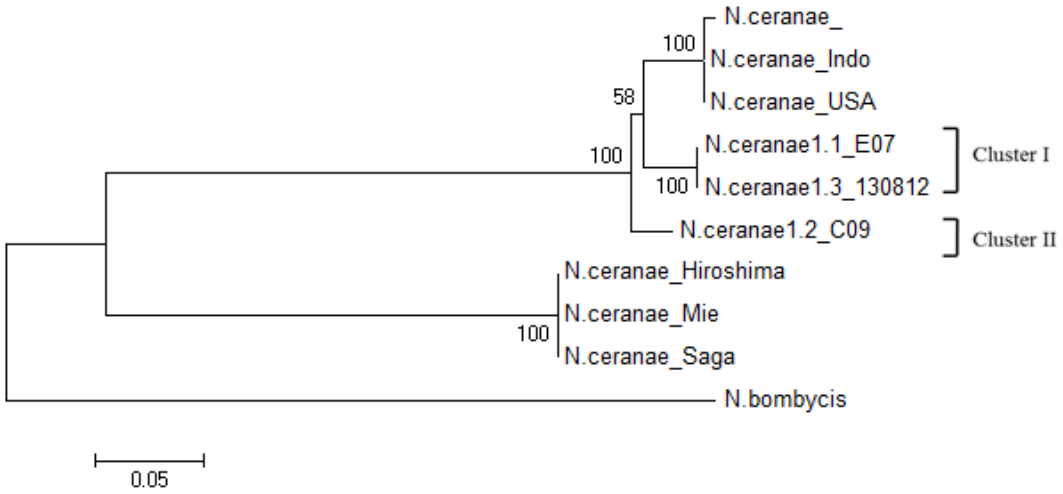

Figure 4.8 An NJ tree of the aligned nucleotide data set of *16S rRNA* gene sequences from *N. ceranae* (200 bp). Three isolates were from Samut-songkhram province while the rest were from other countries. A *16S rRNA* gene sequence of *N. bombycis* was used as an outgroup for the analysis. The number under branches indicates the percentage of bootstrap supported with 1,000 replications.

Interestingly, due to a phylogenetic tree of *16S rRNA* gene in *N. apis*, it revealed that sequences from Samut-songkhram and Chumphon provinces were closely related to the sequence of *Nosema* genus. It exhibited a single distinct clade of cluster I. Thus, the sequences belonged into the same phylogenetic lineage (Figure 4.9). It also showed that the different geographic region of Samut-songkhram and Chumphon did not affect the transfer of genetics. The sequences of *16S rRNA* gene in *N. bombi* and *Encephalitozoon cuniculi* were used as outgroups with 1,000 bootstrap replications. However, the sequence of *N. apis* in cluster I as monophyletic group was closely related to the sequence of *N. bombi* more than the sequence of *E. cuniculi* as in Figure 4.9.

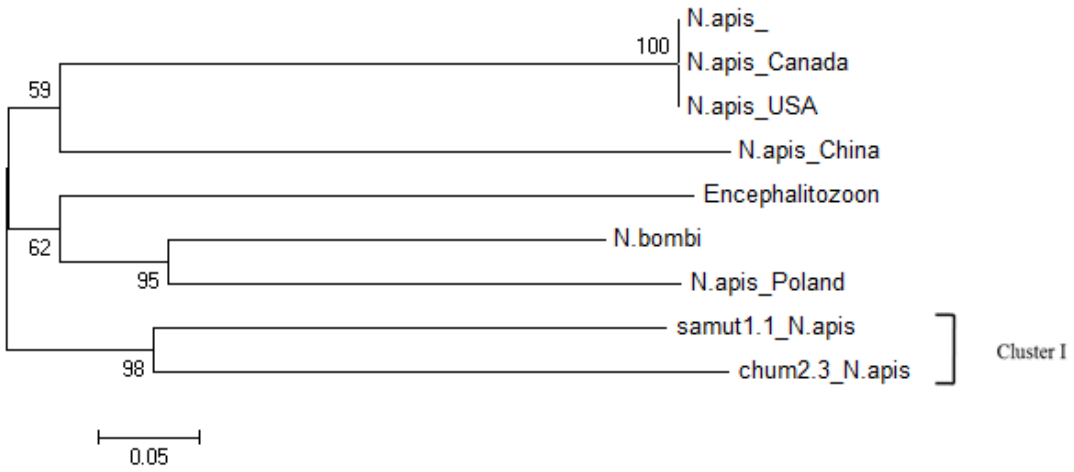

Figure 4.9 An NJ tree of the aligned nucleotide data set of *16S rRNA* gene sequences from *N. apis* (300 bp). Two isolates were from Samut-songkhram and Chumphon provinces while the rest were from other countries. A *16S rRNA* gene sequence of *N. bombi* and *Encephalitozoon cuniculi* were used as outgroups for the analysis. The number under branches indicates the percentage of bootstrap supported with 1,000 replications.

Considering a phylogenetic tree of *5.8S rRNA* gene in *A. apis*, it revealed that the sequence from Chumphon province was closely related to the sequence of *Ascosphaera* genus. It exhibited four distinct clades which were cluster I, II, III, and IV (Figure 4.10). Cluster I contained the sequence of *A. apis* found in palm trees from La-mae district. On the contrary, cluster II, III, and IV contained the sequence of *A. apis* found in rubber, cashew nut, and mango trees, respectively. Furthermore, cluster III and IV were closely related to phylogenetic lineage. The *5.8S rRNA* gene sequence of *Beauveria bassiana* was used as an outgroup with 1,000 bootstrap replications. Therefore, the sequences of *A. apis* in cluster I, II, III, and IV showed common ancestor more than one as paraphyletic group.

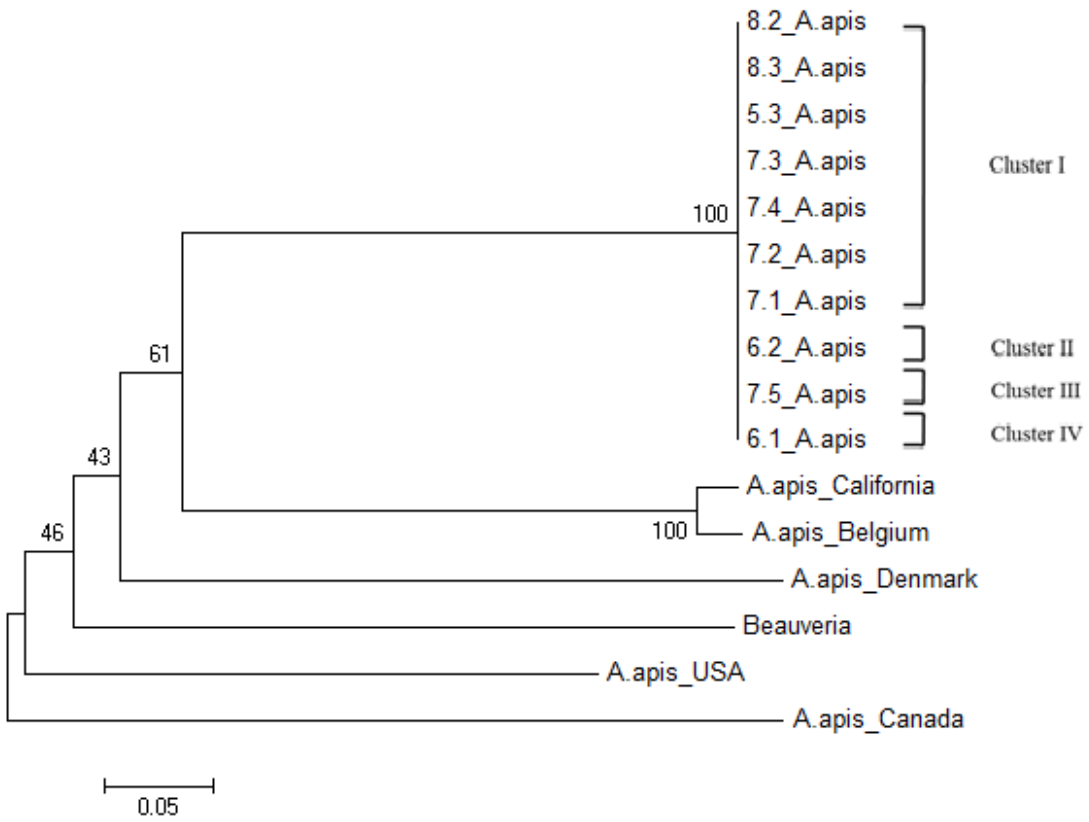

Figure 4.10 An NJ tree of the aligned nucleotide data set of *5.8S rRNA* gene sequences from *A. apis* (500 bp). Ten isolates were from Chumphon province) and the rest were from other countries. A *5.8S rRNA* gene sequence of *Beauveria bassisna* was used as an outgroup for the analysis. The number under branches indicates the percentage of bootstrap supported with 1,000 replications.

4.4 Gut bacteria in healthy bee

4.4.1 Bacterial identification

In order to find a safe way to kill bee pathogens, probiotic is now challenging. At the beginning, aerobic and anaerobic bacteria in healthy bee's gut were isolated and identified. Four methods were used.

The first one was by morphology observation. Most colonies looked yellow and convex with smooth edge at different size. Colony forming unit (CFU)/ml was calculated and recorded as in Table 4.5.

The second one, each colony was identified by gram staining. The result was showed in Figure 4.11 as an example. By this method, not only gram of any bacteria could be seen, but the shape of bacteria could be also observed.

The third method, the partial sequence of *16S rRNA* gene from any colony was amplified under the optimum condition. After agarose gel electrophoresis, the expected product of about 1.4 kb in size could be seen (Figure 4.12). After the expected product was purified, direct sequenced, and searched for similarity, the partial sequence of *16S rRNA* from bacteria could be obtained. An example of the sequence was reported as in Figure 4.13.

The fourth method, the type of carbon sources that bacteria prefer was found out by fermentation test. The result was showed in Tables 4.5 and 4.6, respectively.

Considering Tables 4.5 and 4.6, by the partial sequence of *16S rRNA* analysis of 30 colonies, 10 colonies were identified to be of *Bacillus* sp. Five colonies were *Pantoea* sp. Five colonies were *Azotobacter* sp. Two colonies were *Staphylococcus* sp. Two colonies were *Klebsiella* sp. Two colonies were *Lactobacillus* sp. Two colonies were lactic acid bacteria. One colony was *Escherichia sp.* and one colony was *Shigella* sp. Moreover, the same 30 colonies were repeatedly identified by fermentation. Five colonies were identified to be *Pantoea* sp. Four colonies were *Bacillus* sp. Four colonies were *Bifidobacterium* sp. Four colonies were *Actinomyces* sp. Three colonies were *Photobacter* sp. Two colonies were *Klebsiella* sp. Two colonies were *Fusobacterium* sp. Two colonies were *Clostridium* sp. One colony was *Lactobacillus* sp. One colony was *Staphylococcus* sp. One colony was *Shigella* sp. and one colony was *Bacteroides* sp. Although, the results of identification of some colonies were different by two methods, it seemed that the result of the partial sequence of *16S rRNA* analysis was more reliable. Nonetheless, the same class of taxonomy could be obtained similarly by both methods.

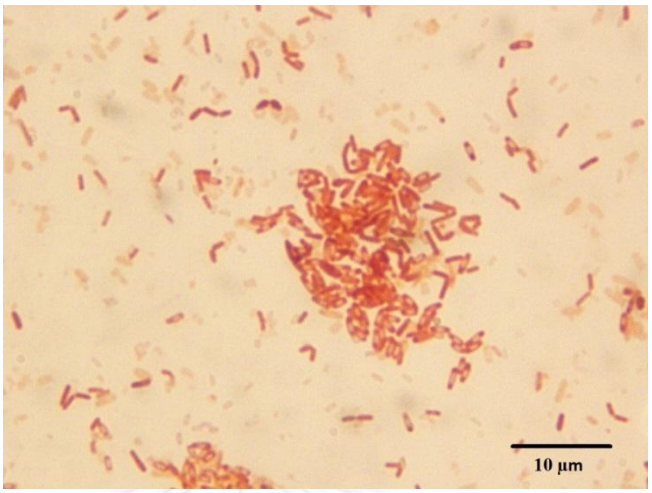

Figure 4.11 Gram stain of aerobic bacteria in larvae's gut from apiary # 2, colony # 1, Samut-songkhram province.

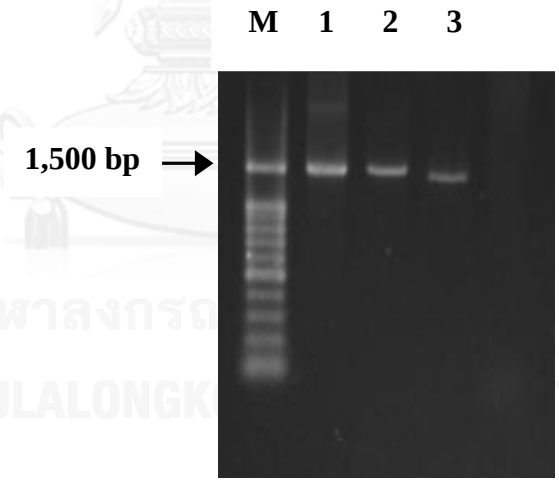

Figure 4.12 Amplified PCR product of *16S rRNA* gene after electrophoresis. Lane M was 100 bp DNA ladder as DNA marker. Lanes 1-3 were amplified PCR products (1.4 kb).

```
Query  30   ACGCTTTTTCTTCCACCGGAGCTTGCTCCACCGGAAGAAAAGGAGTGGCGAACGGGTGAG  89
            ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Genbank39   ACGCTTTTTCTTCCAYCGGAGCTTGCTCCACCGGAAGAAAAGGAGTGGCGAACGGGTGAG  98

Query  90   TAACACGTGGGTAACCTACCCTCGAGCGGGGGATAACACTTGGAAACAGGTGCTAATACC  149
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank99   TAACACGTGGGTAACCTACCCTCGAGCGGGGGATAACACTTGGAAACAGGTGCTAATACC  158

Query  150  GCATAACAAAGAAAACCGCATGGTTTTCTTTTGAAAGGCGCTTTTGCGTCACTCGAGGAT  209
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank159  GCATAACAAAGAAAACCGCATGGTTTTCTTTTGAAAGGCGCTTTTGCGTCACTCGAGGAT  218

Query  210  GGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAG  269
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank219  GGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAG  278

Query  270  CCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGA  329
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank279  CCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGA  338

Query  330  GGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGT  389
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank339  GGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGT  398

Query  390  GAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGGTGAGAGTAAAA  449
            |||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Genbank399  GAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGGTGAGAGTAGAA  458

Query  450  AGTTCACCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC  509
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank459  AGTTCACCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC  518

Query  510  GGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGG  569
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank519  GGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGG  578
```

```
Query  570  TTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGA  629
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Genbank579  TTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGA  638


Query  630  AACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATA  689
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank639  AACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATA  698


Query  690  TGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAA  749
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank699  TGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAA  758


Query  750  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA  809
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank759  GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA  818


Query  810  AGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG  869
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank819  AGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG  878


Query  870  GAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG  929
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank879  GAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG  938


Query  930  CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC  981
            |||||||||||||||||||||||||||||||||||||||||||||||||||
Genbank939  CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC  990
```


Figure 4.13 The partial sequence of *16S rRNA* gene of anaerobic bacteria in larvae's gut from apiary # 1, colony # 1. The obtained sequence was blasted to search for the similarity to the recorded sequences in GenBank database. Here, it was similar to the sequence of *Enterococcus* sp. with 99% dentities and 0% gaps.

Table 4.5 Identification and classification of targeted bacteria in healthy bee's gut.

Bee stage/ bacteria type// apiary #	**Counted colony (CFU/ml)**	**Bacteria in healthy bee's gut**			
		Partial sequence of *16S rRNA*	**Gram stain**	**Shape of bacteria**	**Fermentation**
Larva/ aerobic// 1	2,164 ± 157.30	*Pantoea* sp.	-	Rod	*Pantoea* sp.
Larva/ anaerobic// 1	4,052 ± 43.79	*Enterococcus* sp.	+	Rod	*Bifidobacterium* sp.
Worker/ aerobic// 1	670 ± 41.65	*Pantoea* sp.	-	Rod	*Pantoea* sp.
Worker/ anaerobic// 1	1,218 ± 57.33	*Escherichia* sp.	-	Rod	*Bacteroides* sp.
Larva/ aerobic// 2	475 ± 34.45	*Shigella* sp.	-	Rod	*Shigella* sp.
Larva/ anaerobic// 2	2,731 ± 95.55	*Staphylococcus* sp.	+	Coccus	*Clostridium* sp.
Worker/ aerobic// 2	4,463 ± 16.55	*Pantoea* sp.	-	Rod	*Pantoea* sp.
Worker/ anaerobic// 2	6,390 ± 532.02	*Bacillus* sp.	+	Rod	*Actinomyces* sp.
Larva/ aerobic// 3	775 ± 9.56	*Pantoea* sp.	-	Rod	*Pantoea* sp.
Larva/ anaerobic// 3	820 ± 28.67	*Staphylococcus* sp.	+	Coccus	*Clostridium* sp.

Bee stage/ bacteria type// apiary #	Counted colony (CFU/ml)	Bacteria in healthy bee's gut			
		Partial sequence of *16S rRNA*	Gram stain	Shape of bacteria	Fermentation
Worker/ aerobic// 3	1,125 ± 0.00	*Klebsiella* sp.	-	Rod	*Klebsiella* sp.
Worker/ anaerobic// 3	1,040 ± 9.56	*Bacillus* sp.	+	Rod	*Actinomyces* sp.
Worker/ aerobic// 3.1	1,783 ± 9.07	*Bacillus* sp.	+	Rod	*Brevibacillus* sp.
Worker/ aerobic// 3.2	5,150 ± 2.00	*Bacillus* sp.	+	Rod	*Bacillus* sp.
Worker/ aerobic// 3.3	185 ± 1.15	*Bacillus* sp.	+	Rod	*Aneurinibacillus* sp.
Worker/ aerobic// 3.4	6,183 ± 23.01	*Pantoea* sp.	-	Rod	*Pantoea* sp.
Worker/ aerobic// 3.5	1,350 ± 4.00	*Bacillus* sp.	+	Rod	*Brevibacillus* sp.
Worker/ aerobic// 3.6	300 ± 1.00	*Azotobacter* sp.	-	Rod	*Photobacterium* sp.
Worker/ aerobic// 3.7	450 ± 1.00	*Azotobacter* sp.	-	Rod	*Photobacterium* sp.
Worker/ aerobic// 3.8	1,200 ± 16.26	*Azotobacter* sp.	-	Rod	*Photobacterium* sp.
Worker/ aerobic// 3.9	280 ± 5.03	*Klebsiella* sp.	-	Rod	*Klebsiella* sp.

Bee stage/ bacteria type// apiary #	Counted colony (CFU/ml)	Bacteria in healthy bee's gut			
		Partial sequence of *16S rRNA*	Gram stain	Shape of bacteria	Fermentation
Worker/ anaerobic// 3.1	4,950 ± 2.00	Lactic acid bacteria	+	Rod	*Bifidobacterium* sp.
Worker/ anaerobic// 3.2	5,000 ± 46.23	*Bacillus* sp.	+	Rod	*Bifidobacterium* sp.
Worker/ anaerobic// 3.3	4,800 ± 14.80	*Azotobacter* sp.	-	Rod	*Fusobacterium* sp.
Worker/ anaerobic// 3.4	2,717 ± 35.16	*Lactobacillus* sp.	+	Rod	*Staphylococcus* sp.
Worker/ anaerobic// 3.5	917 ± 3.51	*Lactobacillus* sp.	+	Rod	*Lactobacillus* sp.
Worker/ anaerobic// 3.6	2,450 ± 2.00	Lactic acid bacteria	+	Rod	*Bifidobacterium* sp.
Worker/ anaerobic// 3.7	933 ± 2.52	*Azotobacter* sp.	-	Rod	*Fusobacterium* sp.
Worker/ anaerobic// 3.8	4,417 ± 51.59	*Bacillus* sp.	+	Rod	*Actinomyces* sp.
Worker/ anaerobic// 3.9	2,350 ± 35.93	*Bacillus* sp.	+	Rod	*Actinomyces* sp.

Table 4.6 The percentage of maximal identity of bacteria in the bee's gut sequences to the recorded sequences in GenBank database.

Bacteria	Accession #	Description	Length of identity
Larva/ aerobic// 1	NR_041978.1	*Pantoea agglomerans* strain DSM 3493 *16S ribosomal RNA*, partial sequence	93-97%
Larva/ anaerobic// 1	DQ_411812.1	*Enterococcus pallens* strain ATCC BAA-351 *16S ribosomal RNA* gene, partial sequence	98-99%
Worker/ aerobic// 1	NR_103927.1	*Pantoea ananatis* LMG 20103 strain LMG 20103 *16S ribosomal RNA*, complete sequence	92-96%
Worker/ anaerobic// 1	NR_074902.1	*Escherichia fergusonii* ATCC 35469 strain ATCC 35469 *16S ribosomal RNA*, complete sequence	95-99%
Larva/ aerobic// 2	NR_026331.1	*Shigella flexneri* strain ATCC 29903 *16S ribosomal RNA*, partial sequence	95-99%
Larva/ anaerobic// 2	NR_102499.1	*Staphylococcus warneri* SG1 strain SG1 *16S ribosomal RNA*, complete sequence	93-97%
Worker/ aerobic// 2	NR_104928.1	*Pantoea stewartii* strain CIP 104006 *16S ribosomal RNA*, complete sequence	92-96%

Bacteria	Accession #	Description	Length of identity
Worker/ anaerobic// 2	NR_074453.1	*Bacillus anthracis* str. Ames strain Ames *16S ribosomal RNA*, complete sequence	88-92%
Larva/ aerobic// 3	NR_103927.1	*Pantoea ananatis* strain LMG 20103 *16S ribosomal RNA*, complete sequence	94-98%
Larva/ anaerobic// 3	NR_024669.1	*Staphylococcus pasteuri* strain ATCC51129 *16S ribosomal RNA*, partial sequence	94-98%
Worker/ aerobic// 3	NR_074913.1	*Klebsiella pneumoniae* subsp. strain ATCC 700721 *16S ribosomal RNA*, complete sequence	93-97%
Worker/ anaerobic// 3	NR_043268.1	*Bacillus idriensis* strain SMC 4352-2 *16S ribosomal RNA*, partial sequence	90-91%
Worker/ aerobic// 3.1	NR_043084.1	*Bacillus koreensis* strain BR030 *16S ribosomal RNA*, partial sequence	92-96%
Worker/ aerobic// 3.2	NR_074453.1	*Bacillus anthracis* str. Ames strain Ames *16S ribosomal RNA*, complete sequence	92-94%
Worker/ aerobic// 3.3	NR_043268.1	*Bacillus idriensis* strain SMC 4352-2 *16S ribosomal RNA*, partial sequence	91-95%
Worker/ aerobic// 3.4	NR_103927.1	*Pantoea ananatis 16S rRNA*, complete sequence	92-96%
Worker/ aerobic// 3.5	NR_074914.1	*Bacillus cytotoxicus* NVH 391-98 strain NVH 391-98 *16S ribosomal RNA*, complete sequence	90-94%
Worker/ aerobic// 3.6	NR_041037.1	*Azotobacter armeniacus* strain DSM 2284 *16S ribosomal RNA*, partial sequence	90-94%

Bacteria	Accession #	Description	Length of identity
Worker/ aerobic// 3.7	NR_041039.1	*Azotobacter vinelandii* strain IAM 15004 *16S ribosomal RNA*, partial sequence	89-92%
Worker/ aerobic// 3.8	NR_041033.1	*Azotobacter nigricans* subsp. nigricans strain IAM 15005 *16S ribosomal RNA*, partial sequence	89-91%
Worker/ aerobic// 3.9	NR_074729.1	*Klebsiella variicola* At-22 strain At-22 *16S ribosomal RNA*, complete sequence	93-97%
Worker/ anaerobic// 3.1	KC748442.1	Lactic acid bacterium ZJGS0113 *16S ribosomal RNA gene*, partial sequence	92-96%
Worker/ anaerobic// 3.2	NR_043334.1	*Bacillus niabensis* strain 4T19 *16S ribosomal RNA*, partial sequence	94-98%
Worker/ anaerobic// 3.3	NR_041033.1	*Azotobacter nigricans* subsp. nigricans strain IAM 15005 *16S ribosomal RNA*, partial sequence	88-90%
Worker/ anaerobic// 3.4	NR_028658.1	*Lactobacillus satsumensis* strain NRIC 0604 *16S ribosomal RNA*, complete sequence	90-94%
Worker/ anaerobic// 3.5	NR_041655.1	*Lactobacillus capillatus* strain YIT 11306 *16S ribosomal RNA*, partial sequence	92-96%
Worker/ anaerobic// 3.6	KC748433.1	Lactic acid bacterium ZJGS0609 *16S ribosomal RNA gene*, partial sequence	93-97%
Worker/ anaerobic// 3.7	NR_041039.1	*Azotobacter vinelandii* strain IAM 15004 *16S ribosomal RNA*, partial sequence	88-90%
Worker/ anaerobic// 3.8	NR_074914.1	*Bacillus cytotoxicus* NVH 391-98 strain NVH 391-98 *16S ribosomal RNA*, complete sequence	90-95%
Worker/ anaerobic// 3.9	NR_074540.1	*Bacillus cereus* ATCC 14579 strain ATCC 14579 *16S ribosomal RNA*, complete sequence	90-92%

4.5 Antagonistic effect of gut bacteria on AFB

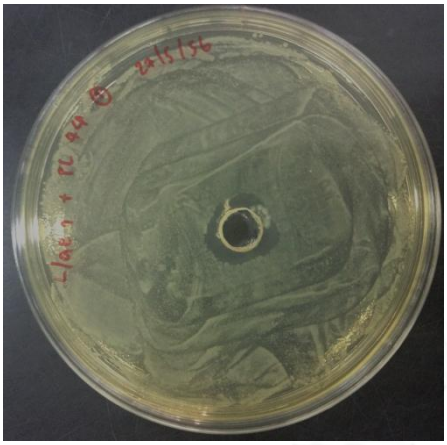
The antagonistic effect of selected gut bacteria which were *Enterococcus* sp., *Bacillus* sp., *Pantoea* sp., *Azotobacter* sp., *Klebsiella* sp., and *Lactobacillus* sp. against *P. larvae* strain 01 and strain 02 was determined by well diffusion. A diameter of relative inhibition zone (%) was recorded with the data that these bacteria were from different stages, oxygen consumption or not, and colony number. For strain 01, the inhibition zone generated by larva/ aerobic// 1, larva/ anaerobic// 1, worker/ aerobic// 3, worker/ aerobic// 3.2, worker/ aerobic// 3.4, worker/ aerobic// 3.6, and worker/ anaerobic// 3.2 were $26.67^{a} \pm 2.17\%$, $42.84^{a} \pm 17.17\%$, $30.51^{b} \pm 13.84\%$, $21.17^{a} \pm 3.50\%$, $54.51^{c} \pm 3.17\%$, $35.51^{b} \pm 7.17\%$, and $35.51^{b} \pm 8.84\%$, respectively.

Whereas, for strain 02, the relative inhibition zone (%) generated by larva/ aerobic// 1, larva/ anaerobic// 1, worker/ aerobic// 1, worker/ aerobic// 2, worker/ aerobic// 3.1, worker/ aerobic// 3.3, worker/ aerobic// 3.4, worker/ aerobic// 3.5, and worker/ aerobic// 3.6 were $26.01^{a} \pm 2.17\%$, $40.51^{b} \pm 17.17\%$, $25.51^{a} \pm 6.67\%$, $27.84^{a} \pm 2.83\%$, $21.67^{a} \pm 6.17\%$, $32.84^{b} \pm 10.34\%$, $56.68^{c} \pm 3.17\%$, $35.51^{b} \pm 8.01\%$, and $45.01^{b} \pm 7.17\%$, respectively. Due to Table 4.7 and Figure 4.14, it revealed that gut bacteria or worker/ aerobic// 3.4 which was later identified as *Azotobacter* sp. showed the highest inhibitory effect on strain 02. The variance difference was significant at the 0.05 level (*p*-value: 0.000).

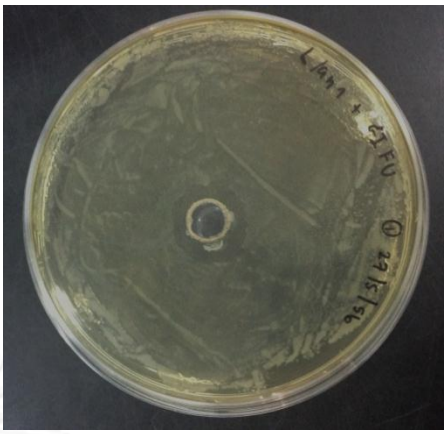
Table 4.7 The antagonistic effect of gut bacteria on AFB.

Bacteria (stage/ condition// apiary #) + strain of *P. larvae*	**Analysis by *16S rRNA* sequence**	**Relative inhibition zone (%)**
Larva/ aerobic// 1+ strain 01	*Pantoea* sp.	$26.67^{a} \pm 2.17$
Larva/ aerobic// 1+ strain 02	*Pantoea* sp.	$26.01^{a} \pm 2.17$
Larva/anaerobic// 1+ strain 01	*Enterococcus* sp.	$42.84^{b} \pm 17.17$
Larva/ anaerobic// 1+ strain 02	*Enterococcus* sp.	$40.51^{b} \pm 17.17$
Worker/ aerobic// 1 + strain 02	*Bacillus* sp.	$25.51^{a} \pm 6.67$
Worker/ aerobic// 2 + strain 02	*Bacillus* sp.	$27.84^{a} \pm 2.83$
Worker/ aerobic// 3 + strain 01	*Bacillus* sp.	$30.51^{b} \pm 13.84$
Worker/ aerobic// 3.1 + strain 02	*Pantoea* sp.	$21.67^{a} \pm 6.17$
Worker / aerobic// 3.2 + strain 01	*Bacillus* sp.	$21.17^{a} \pm 3.50$
Worker/ aerobic// 3.3 + strain 02	*Azotobacter* sp.	$32.84^{b} \pm 10.34$
Worker/ aerobic// 3.4 + strain 01	*Azotobacter* sp.	$54.51^{c} \pm 3.17$
Worker / aerobic// 3.4 + strain 02	*Azotobacter* sp.	$56.68^{c} \pm 3.17$
Worker/ aerobic// 3.5 + strain 02	*Azotobacter* sp.	$35.51^{b} \pm 8.01$
Worker/ aerobic// 3.6 + strain 01	Klebsiella sp.	$35.51^{b} \pm 7.17$
Worker/ aerobic// 3.6 + strain 02	Klebsiella sp.	$45.01^{b} \pm 7.17$
Worker/ anaerobic// 3.2 + strain 01	Lactobacillus sp.	$35.51^{b} \pm 8.84$

[a, b, c] The variance difference was significant at the 0.05 level (One way ANOVA, Post hoc test: LSD, *p*-value: 0.000).



(A)



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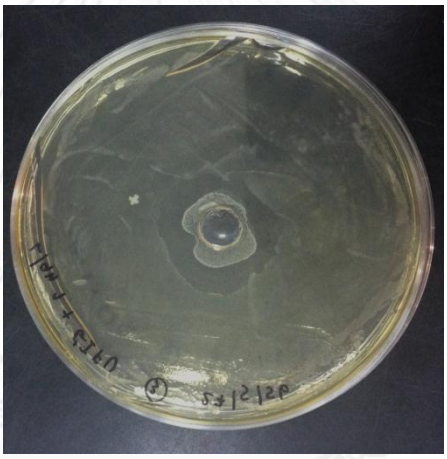

(C)

Figure 4.14 Antagonistic effect against *P. larvae*. *Pantoea* sp. from larva/ aerobic// 1 could inhibit strain 02 (A) and strain 01 (B) while *Enterococcus* sp. from larva/ anaerobic// 1 could inhibit strain 01 (C).

4.6 Antibiotic resistance of the isolated *P. larvae* strain 01 and strain 02

Various antibiotics such as nalidixic acid, streptomycin, tetracycline, penicillin, and ampicillin were used. Inhibition zone was measured by disc diffusion method. Minimum inhibitory concentration (MIC) of antibiotics was determined by using serial dilution method. The result showed that both strain 01 and strain 02 were resistant to streptomycin and nalidixic acid. For strain 02, the same MIC value of streptomycin and nalidixic acid were 0.3^{a} µg/ml. The inhibition zones were 0.9 cm (for streptomycin) and 1.4 cm (for nalidixic acid), respectively. The mean difference was significant at the 0.05 level (*p*-value: 0.006). In addition, for strain 01, the MIC value of streptomycin was 0.6^{b} µg/ml and the MIC value of nalidixic acid was 0.7^{c} µg/ml, respectively. The inhibition zones were 1.1 cm (for streptomycin) and 0.9 cm (for nalidixic acid), respectively. The mean difference was significant at the 0.05 level (*p*-value: 0.001). All data was recorded in Table 4.8 and Figure 4.15.

Table 4.8 Minimum inhibition concentration (MIC) of antibiotics to *P. larvae*.

Antibiotic	*P. larvae* strain	MIC (µg/ml)
Streptomycin	02	0.3[a]
Nalidixic acid	02	0.3[a]
Streptomycin	01	0.6[b]
Nalidixic acid	01	0.7[c]

[a, b] The superscript lowercase letter presents the significance of mean difference at the 0.05 level (One way ANOVA, Post hoc test: LSD, *p*-value: 0.006) and [a, c] The superscript lowercase letter presents the significance of mean difference at the 0.05 level (One way ANOVA, Post hoc test: LSD, *p*-value: 0.001).

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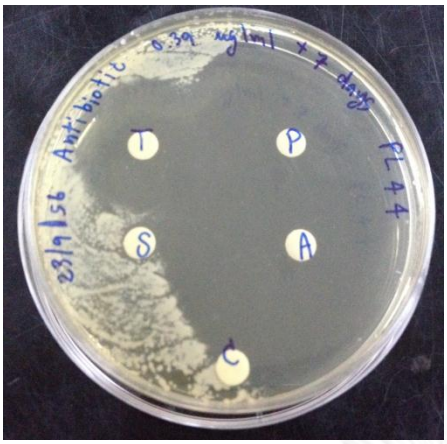
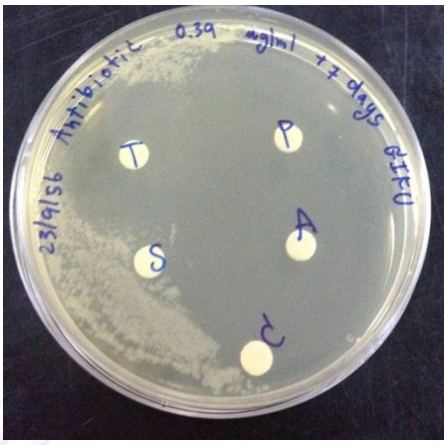


(A)



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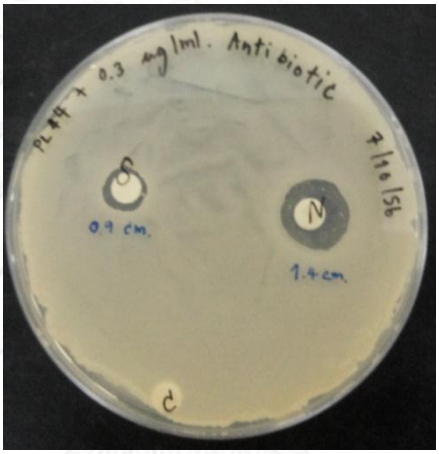

(C)

Figure 4.15 Antibiotic sensitivity of *P. larvae*, strain 02 (A and C) and strain 01 (B). C on agar was control. A on agar was ampicillin. N was nalidixic acid. S was streptomycin and T was tetracycline. The concentration used in each disc was 0.3[a] µg/ml.

4.7 Antibacterial activity of gut bacteria's supernatant

The supernatant of targeted bacteria was also tested for inhibitory effect on the growth of strain 01 and strain 02. It showed that antibacterial activities against strain 01 and strain 02 at 37 °C were from *Bacillus* sp., *Lactobacillus* sp., *Klebsiella* sp., and *Azotobacter* sp. After 72 h, the percentage of strain 01 and strain 02 growth after treated with the supernatant was in the range of 29.75^{a} ± 9.35% to 80.54^{b} ± 42.84%. The result showed that *Lactobacillus* sp. and *Azotobacter* sp. could efficiently reduce the percentage of strain 01 and strain 02 growth at about 29.75^{a} ± 9.35% (Table 4.9) and 61.14^{c} ± 21.13% (Table 4.10), respectively. In addition, *Lactobacillus* sp. revealed the highest inhibitory activity which the mean difference was significant at the 0.05 level (*p*-value: 0.010). Besides, supernatants which could inhibit both strain 01 and strain 02 were from *Bacillus* sp. (74.36^{b} ± 17.14% and 66.24^{a} ± 24.98%) and *Azotobacter* sp. (65.22^{b} ± 15.53% and 80.54^{a} ± 42.84%) in accordance as in Tables 4.9 and 4.10. The mean difference of percentage of strain 01 and strain 02 growth was not significant at the 0.05 level (*p*-value: 1.000). In addition, *Klebsiella* sp. was able to inhibit strain 01 only which the mean difference of percentage of growth at 72 h (about 89.08^{a} ± 0.81%) was still not significant at the 0.05 level (*p*-value: 1.000). Moreover, *Bacillus* sp. and *Azotobacter* sp. could inhibit strain 01 and strain 02 insignificantly.

Table 4.9 Antibacterial activity of gut bacteria's supernatant at 37 °C on strain 01.

Supernatant from	% Growth of treated strain 01		
	24 h	**48 h**	**72 h**
W/ae//3.2 (*Bacillus* sp.)	136.38 ± 32.92	109.32 ± 29.51	74.36[b] ± 17.14
W/ae//3.4 (*Azotobacter* sp.)	126.42 ± 30.51	125.94 ± 22.74	65.22[b] ± 15.53
W/ae//3.6 (*Klebsiella* sp.)	172.27 ± 2.38	110.92 ± 14.50	89.08[b] ± 0.81
W/an//3.2 (*Lactobacillus* sp.)	148.76 ± 1.17	38.43 ± 22.79	29.75[a] ± 9.35
Positive control (strain 01)	0.00	52.54	128.81[c]

The superscript lowercase letter presents the significance of mean difference at the 0.05 level (One way ANOVA, Post hoc test: LSD, *p*-value: 0.000).

[a, b] One way ANOVA, Post hoc test: LSD, *p*-value: 0.010; [a, c] One way ANOVA, Post hoc test: LSD, *p*-value: 0.004

Table 4.10 Antibacterial activity of gut bacteria's supernatant at 37 °C on strain 02.

Supernatant from	**% Growth of treated strain 02**		
	24 h	**48 h**	**72 h**
W/ae//2 (*Bacillus* sp.)	175.96 ± 1.36	169.53 ± 1.64	66.24[a] ± 24.98
W/ae//3.3 (*Azotobacter* sp.)	147.25 ± 14.94	125.52 ± 14.73	61.14[a] ± 21.13
W/ae//3.4 (*Azotobacter* sp.)	152.34 ± 11.05	150.00 ± 11.05	80.54[a] ± 42.84
Positive control (strain 02)	0.00	81.50	102.31[b]

The superscript lowercase letter presents the significance of mean difference at the 0.05 level (One way ANOVA, Post hoc test: LSD, *p*-value: 0.000).

[a, b] One way ANOVA, Post hoc test: LSD, *p*-value: 0.484

4.8 Effect of heat treatment on supernatant to antibacterial activity

The supernatant of targeted bacteria treated at 90 °C for 1 h was also tested for inhibitory effect on the growth of strain 01 and strain 02. It showed that antibacterial activities against both strains were from *Bacillus* sp., *Lactobacillus* sp., *Klebsiella* sp., and *Azotobacter* sp. Within 72 h, the percentage of strain 01 and strain 02 growth was in the range of 0.00^{a} % to 43.42^{b} ± 2.58% (Tables 4.11 and 4.12). The result showed that *Lactobacillus* sp. and *Bacillus* sp. could efficiently reduce the percentage of strain 01 (0.00^{a} %) and strain 02 (6.41^{a} ± 7.44%) growth. In overall, *Lactobacillus* sp. (0.00^{a} %), *Klebsiella* sp. (12.48^{a} ± 1.06%), *Azotobacter* sp. (8.75^{a} ± 2.10%), and *Bacillus* sp. (6.41^{a} ± 7.44%) revealed the highest inhibitory activity which the mean difference was not significant at the 0.05 level (*p*-value: 0.154). Besides, the *Bacillus* sp. supernatant treated at 90 °C could inhibit the growth of both strain 01 (43.42^{b} ± 2.58%) and strain 02 (6.41^{a} ± 7.44%) which the mean difference was not significant at the 0.05 level (*p*-value: 0.238). Besides, *Pantoea* sp. could inhibit the growth of strain 02 only which the mean difference of the percentage of growth at 72 h (about 30.67^{b} ± 8.79%) still remained not significant at the 0.05 level (*p*-value: 0.238). The mean difference between group “a” and “b” was not significant (*p*-value: 0.238) at the 0.05 level.

However, the supernatant treated at 121 °C did not show the inhibitory effect against strain 01 and strain 02 at all. At 72 h incubation, the percentage of strain 01 and strain 02 growth was still increased, comparing to positive control (data not shown).

Table 4.11 The antibacterial activity against strain 01 of supernatant treated at 90 °C for 1 h.

Supernatant from	% Growth of treated strain 01		
	24 h	48 h	72 h
W/ae//3.2 (*Bacillus* sp.)	63.49 ± 3.16	48.41 ± 5.61	43.42[b] ± 2.58
W/an//3.2 (*Lactobacillus* sp.)	28.58 ± 2.64	8.32 ± 7.31	0.00[a]
Positive control (strain 01)	0.00	20.69	50.86[b]

The superscript lowercase letter presents the insignificance of mean difference at the 0.05 level.

[a, b] One way ANOVA, Post hoc test: LSD, *p*-value: 0.238

Table 4.12 The antibacterial activity against strain 02 of supernatant treated at 90 °C for 1 h.

Supernatant from	% Growth of treated strain 02		
	24 h	48 h	72 h
W/ae//2 (*Bacillus* sp.)	44.03 ± 6.76	34.88 ± 16.65	6.41[a] ± 7.44
W/ae//3.1 (*Pantoea* sp.)	45.19 ± 2.14	34.84 ± 2.71	30.67[b] ± 8.79
W/ae//3.3 (*Azotobacter* sp.)	42.23 ± 9.68	19.53 ± 9.30	8.75[a] ± 2.10
W/ae//3.6 (*Klebsiella* sp.)	64.46 ± 9.40	24.46 ± 7.55	12.48[a] ± 1.06
Positive control (strain 02)	2.11	34.18	65.49[c]

The superscript lowercase letter presents the insignificance of mean difference at the 0.05 level.

[a, b] One way ANOVA, Post hoc test: LSD, *p*-value: 0.238

The superscript lowercase letter presents the significance of mean difference at the 0.05 level.

[a, c] One way ANOVA, Post hoc test: LSD, *p*-value: 0.010

4.9 Effect of enzymes on supernatant to antibacterial activity

The targeted bacterial supernatant was also treated with proteinase K and RNase A. After 72 h incubation, the percentage of strain 01 and strain 02 growth was still increased, comparing to positive control. Therefore, the supernatant potential was affected by proteinase K and RNase A at all (data not shown).

4.10 Effect of pH on cell free supernatant to antibacterial activity

The supernatant of targeted bacteria was treated with 3 drops of 0.1 N HCl to adjust the pH to be 5.0. Also, the supernatant was treated with 5 drops of 0.1 N HCl to adjust the pH to be 3.0. Due to tables 4.13-4.16, the active supernatant was from *Pantoea* sp., *Bacillus* sp., and *Klebsiella* sp. The percentage of strain 01 and strain 02 growth within 72 h was in the range of 0.00^{a} % to 73.96^{b} ± 8.67%. It showed that the *Pantoea* sp. supernatant at pH 5 and the *Bacillus* sp. supernatant at pH 3 could efficiently reduce the percentage of strain 01 (0.00^{a} %) and strain 02 (10.92^{a} ± 17.38%) growth. In overall, the supernatant from *Pantoea* sp. at pH 5 (0.00^{a}% for strain 01 and 5.45^{a} ± 1.34% for strain 02), *Bacillus* sp. at pH 3 (23.48^{a} ± 13.93% for strain 01 and 10.92^{a} ± 17.38% for strain 02), and *Klebsiella* sp. at pH 3 (45.04^{b} ± 15.27% for strain 01) revealed the highest inhibitory activity which the mean difference was not significant at the 0.05 level (*p*-value: 0.238). In addition, the treated supernatant that could inhibit both strain 01 and strain 02 was from *Pantoea* sp. at any pH. However, the supernatant from *Bacillus* sp. was active to both strains at pH 3 only (23.48^{a} ± 13.93% for strain 01 and 10.92^{a} ± 17.38% for strain 02) which the mean difference of the percentage of strain 01 and strain 02 growth was not significant at the 0.05 level (*p*-value: 0.154). In Table 4.15, the supernatant from *Klebsiella* sp. and *Bacillus* sp. at pH 3 could inhibit strain 01 only which the mean difference of the percentage of growth at 72 h (about 45.04^{b} ± 15.27% for *Klebsiella* sp. and 73.96^{c} ± 8.67% for *Bacillus* sp.) still remained not significant at the 0.05 level (*p*-value: 0.315). The mean difference between group “a” and “b” (*p*-value: 0.238) was not significant at the 0.05 level. Also, the mean difference between group “a” and “c” (*p*-value: 0.010) was significant at the 0.05 level.

Table 4.13 The antibacterial activity against strain 01 of treated cell free supernatant at pH 5.

Supernatant from	% Growth of treated strain 01		
	24 h	48 h	72 h
L/ae//1 (*Pantoea* sp.)	64.03 ± 14.72	62.58 ± 13.40	0.00[a]
Positive control (strain 01)	0.00	81.18	90.43[b]

The superscript lowercase letter presents the significance of mean difference at the 0.05 level.

[a, b] One way ANOVA, Post hoc test: LSD, *p*-value: 0.014

Table 4.14 The antibacterial activity against strain 02 of treated cell free supernatant at pH 5.

	% Growth of treated strain 02		
Supernatant from	**24 h**	**48 h**	**72 h**
L/ae//1 (*Pantoea* sp.)	60.68 ± 7.66	25.85 ± 0.59	5.45[a] ± 1.34
Positive control (strain 02)	0.00	95.24	98.85[b]

The superscript lowercase letter presents the significance of mean difference at the 0.05 level.

[a, b] One way ANOVA, Post hoc test: LSD, *p*-value: 0.014

Table 4.15 The antibacterial activity against strain 01 of treated cell free supernatant at pH 3.

Supernatant from	% Growth of treated strain 01		
	24 h	48 h	72 h
L/ae//1 (*Pantoea* sp.)	91.69 ± 4.37	62.58 ± 27.54	0.00[a]
W/ae//3 (*Bacillus* sp.)	111.91 ± 2.44	74.33 ± 2.26	23.48[a] ± 13.93
W/ae//3.2 (*Bacillus* sp.)	115.40 ± 8.25	95.84 ± 37.16	73.96[c] ± 8.67
W/ae//3.6 (*Klebsiella* sp.)	101.89 ± 9.11	67.65 ± 5.68	45.04[b] ± 15.27
Positive control (strain 01)	3.45	33.64	133.08[d]

The superscript lowercase letter presents the insignificance of mean difference at the 0.05 level.

[a, b] One way ANOVA, Post hoc test: LSD, *p*-value: 0.238 and [c, d] One way ANOVA, Post hoc test: LSD, *p*-value: 0.484

The superscript lowercase letter presents the significance of mean difference at the 0.05 level.

[a, c] One way ANOVA, Post hoc test: LSD, *p*-value: 0.010 and [a, d] One way ANOVA, Post hoc test: LSD, *p*-value: 0.014

Table 4.16 The antibacterial activity against strain 02 of treated cell free supernatant at pH 3.

Supernatant from	% Growth of treated strain 02		
	24 h	48 h	72 h
L/ae//1 (*Pantoea* sp.)	44.83 ± 3.40	35.19 ± 7.77	0.00[a]
W/ae//1 (*Bacillus* sp.)	54.91 ± 7.66	37.29 ± 5.80	10.92[a] ± 17.38
W/ae//2 (*Bacillus* sp.)	57.85 ± 3.78	53.42 ± 5.55	43.70[b] ± 12.84
Positive control (strain 02)	2.22	24.17	76.53[c]

The superscript lowercase letter presents the insignificance of mean difference at the 0.05 level.

[a, b] One way ANOVA, Post hoc test: LSD, *p*-value: 0.238

The superscript lowercase letter presents the significance of mean difference at the 0.05 level.

[a, c] One way ANOVA, Post hoc test: LSD, *p*-value: 0.010

Moreover, the supernatant was treated with 3, 5, and 6 drops of 0.2 N NaOH to adjust the pH to be 8, 9, and 10. The result was recorded in Tables 4.17-4.20. The treated supernatant from *Lactobacillus* sp., *Bacillus* sp., *Azotobacter* sp., and *Klebsiella* sp. showed the antibacterial activity against strain 01 and strain 02. In overall, the percentage of strain 01 and strain 02 growth within 72 h was in the range of 529.15^{a} ± 45.91% to $1,021.60^{b}$ ± 39.53%. It showed that the treated supernatant of *Klebsiella* sp. at pH 10 and of *Azotobacter* sp. at pH 10 could efficiently reduce the percentage of strain 01 and strain 02 growth at about 529.15^{a} ± 45.91% (Table 4.19) and $1,008.73^{b}$ ± 53.65% (Table 4.20), respectively. Furthermore, the treated supernatant of *Lactobacillus* sp. at pH 8 (532.67^{a} ± 44.96%, Table 4.17) and pH 10 (536.15^{a} ± 22.83%, Table 4.19) and of *Klebsiella* sp. at pH 10 (529.15^{a} ± 45.91%, Table 4.19) revealed the highest inhibitory activity which mean difference was insignificant at the 0.05 level (*p*-value: 1.000). Furthermore, the treated supernatant (pH 10) which could inhibit both strain 01 ($1,021.60^{b}$ ± 39.53%, Table 4.19) and strain 02 ($1,202.45^{c}$ ± 69.87%, Table 4.20) was from *Bacillus* sp. The mean difference of the percentage of strain 01 and strain 02 growth was significant at the 0.05 level (*p*-value: 0.000). Besides, the treated supernatant from *Azotobacter* sp. (pH 10) could inhibit the growth of strain 01 (883.20^{a} ± 32.53%, Table 4.19) and strain 02 ($1,008.73^{b}$ ± 53.65%, Table 4.20) which the mean difference of the percentage of strain 01 and strain 02 growth was insignificant at the 0.05 level (*p*-value: 1.000). In addition, the treated supernatant from *Bacillus* sp. (pH 10) and from *Lactobacillus* sp. (pH 8 and 9) could inhibit the growth of strain 01 at $1,021.60^{b}$ ± 39.53%, 532.67^{a} ± 44.96%, and 540.43^{a} ± 59.37%, in accordance as in Tables 4.17-4.19. The mean difference of the percentage of growth at 72 h showed that the difference between group “a” and group “b” was significant at the 0.05 level (*p*-value: 0.000). The mean difference between group “a” and group “c” was significant at the 0.05 level (*p*-value: 0.000).

On the other hand, the treated supernatant at pH 8 and 9 did not show the inhibitory effect against strain 02. Within 72 h incubation, the percentage of strain 02 growth was still high, comparing to positive control.

Table 4.17 The antibacterial activity against strain 01 of treated cell free supernatant at pH 8.

Supernatant from	% Growth of treated strain 01		
	24 h	**48 h**	**72 h**
W/an//3.2 (*Lactobacillus* sp.)	813.58 ± 18.10	641.77 ± 16.90	532.67[a] ± 44.96
Positive control (strain 01)	232.76	568.10	971.38[b]

The superscript lowercase letter presents the significance of mean difference at the 0.05 level.

[a, b] One way ANOVA, Post hoc test: LSD, *p*-value: 0.000

Table 4.18 The antibacterial activity against strain 01 of treated cell free supernatant at pH 9.

Supernatant from	% Growth of treated strain 01		
	24 h	48 h	72 h
W/ae//3.2 (*Bacillus* sp.)	1,737.50 ± 20.70	1,767.75 ± 19.03	1,537.28[c] ± 38.20
W/an//3.2 (*Lactobacillus* sp.)	1,307.83 ± 17.22	1,243.91 ± 58.41	540.43[a] ± 59.37
Positive control (strain 01)	29.41	76.47	1,014.29[b]

The superscript lowercase letter presents the significance of mean difference at the 0.05 level.

[a, b, c] One way ANOVA, Post hoc test: LSD, *p*-value: 0.000

Table 4.19 The antibacterial activity against strain 01 of treated cell free supernatant at pH 10.

Supernatant from	% Growth of treated strain 01		
	24 h	**48 h**	**72 h**
W/ae//3.2 (*Bacillus* sp.)	1,309.83 ± 116.05	1,202.68 ± 107.05	1,021.60[b] ± 39.53
W/ae//3.4 (*Azotobacter* sp.)	1,339.74 ± 58.34	1,172.93 ± 96.13	883.20[b] ± 32.53
W/ae//3.6 (*Klebsiella* sp.)	1,199.27 ± 61.46	920.50 ± 77.34	529.15[a] ± 45.91
W/an//3.2 (*Lactobacillus* sp.)	1,383.64 ± 26.85	682.15 ± 53.05	536.15[a] ± 22.83
Positive control (strain 01)	714.21	1,100.41	1,236.08[c]

The superscript lowercase letter presents the significance of mean difference at the 0.05 level.

[a, b, c] One way ANOVA, Post hoc test: LSD, *p*-value: 0.000

Table 4.20 The antibacterial activity against strain 02 of treated cell free supernatant at pH 10.

Supernatant from	% Growth of treated strain 02		
	24 h	48 h	72 h
W/ae//1 (*Bacillus* sp.)	1,422.11 ± 66.94	1,332.69 ± 58.53	1,202.45[b] ± 69.87
W/ae//3.4 (*Azotobacter* sp.)	1,312.35 ± 3.21	1,202.00 ± 26.77	1,198.90[b] ± 20.20
W/ae//3.5 (*Azotobacter* sp.)	1,232.77 ± 10.22	1,044.68 ± 33.85	1,008.73[a] ± 53.65
Positive control (strain 02)	857.39	1,189.57	1,226.09[b]

The superscript lowercase letter presents the significance of mean difference at the 0.05 level.

[a, b] One way ANOVA, Post hoc test: LSD, *p*-value: 0.000

CHAPTER V
DISCUSSION

Bees are economic insects. Not only they are important in crop pollination but their products are also useful in term of food and traditional medicine [1]. Obviously, bee products can give a good income to bee farmers. However, there are a lot of bee diseases which can cause a lot of death to bees. That leads to the economic loss to bee farmers. The main problem is because a symptom is always too severe before a bee farmer can protect or cure the bees. In addition, since bees are eusocial which many thousands of bees live very closely within the same hive, it is very easy for a pathogen to get spread. Thus, it should be much better if we can find a way to know an infection at the very early stage. PCR was undoubtedly introduced to detect the pathogen contamination both in a bee body and in honey. Besides, RNA virus causing a bee disease could be detected by RT-PCR. Later, a portable RT-PCR thermal cycler was successfully developed to work in apiaries in Korea. This kit could be applied to detect many contaminated pathogens. The whole process could be finished within 7.54 min [88].

After diagnosing by multiplex PCR, the number of infected colonies could be seen in Table 4.3. Colonies, from Samut-songkhram province, which were infected by *P. larvae*, *N. ceranae*, and *N. apis* were 4%, 6%, and 2%, respectively. Furthermore, colonies, from Chumphon province, which were infected by *P. larvae*, *N. apis*, and *A. apis* were 16%, 2%, and 20%, respectively. The number of infected colonies in this research was lower than Lauro et al. [89] which reported that 91.07% and 57.14% of *A. mellifera* honey and colonies were positive for *P. larvae* detection, respectively, by using PCR. According to the compared data, it was possible that the susceptibility of pathogen depended on species of bees [89]. As known, *A. mellifera,* an imported species, is very good at foraging and well managed in an apiary. However it is very weak in disease resistance. In contrast, although *A. cerana*, a native species, is a little bit worse in foraging and absconds easily, it is very tolerant to diseases. It can be possible that native species can be well co-evolved to pathogens [90].

Very interesting, *Varroa* mite causing a serious bee problem could get infected by bee pathogens as well. Chantawannakul et al. [60] reported that 5 out of 7 bee virus could be detected in 20 mites, *Varroa destructor,* by using RT-PCR. It was infected by sacbrood bee virus (SBV), Kashmir bee virus (KBV), acute paralysis virus (APV), deformed wing virus (DWV), and black queen cell virus (BQCV). Thus, the crossed species of host for pathogen distribution was possible.

Moreover, the disease infection depends on regions. It was reported that more infection was found in bees from the northern part of Thailand, comparing to this research. Chaimanee et al. [91] observed infection of *N. ceranae* and *N. apis* in *A. dorsata*, *A. cerana*, *A. florea*, and *A. mellifera* which colonies were collected from apiaries and forests in Chiang Mai, Chiang Rai, Lampang, Lamphun, Nan, Phayao, and Phrae provinces from northern Thailand [91]. They revealed that 62 out of 80 colonies of *A. mellifera* (77.5%) were infected by *N. ceranae*. In addition, the native honeybees (*A. cerana*, *A. florea*, and *A. dorsata*) showed that 6 out of 27 (22.2%), 5 out of 11 (45.4%), and 3 out of 8 (37.5%) colonies of those bees were infected by *N. ceranae*, respectively. The prevalence of *N. ceranae* infection in the native honeybees was lower than in *A. mellifera*. However, infection by *N. apis* was not found.

Moreover, it was obvious that nosema disease in bees had been a severe problem over the world. The higher infection of *N. ceranae* in *A. cerana* [92] was found in China (11 out of 18 colonies or 61%), Taiwan (35 out of 48 colonies or 73%), Japan (9 out of 12 colonies or 75%), Indonesia (1 out of 38 colonies or 2.63%), Vietnam (1 out of 38 colonies or 2.63%), South Korea (1 out of 38 colonies or 2.63%), and Solomon islands (8 out of 38 colonies or 21%), respectively [93].

Considering a phylogenetic tree of *16S rRNA* gene in *P. larvae*, *N. ceranae*, *N. apis*, and *5.8S rRNA* gene in *A. apis,* it revealed that the obtained sequences were closely related to the recorded sequences in genus *Paenibacillus*, *Nosema*, and *Ascosphaera* (Figures 4.3-4.6). The phylogenetic trees exhibited 6, 2, 1, and 4 clades, respectively (Figures 4.7-4.10). That presented they were very closely related in phylogenetic lineage. Moreover, considering a phylogenetic analysis of the partial polar tube protein (PTP) gene sequences of *N. ceranae*, it revealed that *N. ceranae* isolated from *A. mellifera* and *A. cerana* were similar in phylogenetic lineage. In contrast, *N.*

ceranae isolated from *A. dorsata* and *A. florea* had 7 and 22 nucleotides difference. Thus, they were clustered into two distinct clades [94].

Infection by *N. apis* in *A.cerana* colonies in China, Taiwan, and Japan was reported to be 28%, 33%, and 25%, respectively. The phylogenetic lineage based on the partial sequences of *SSUrRNA* of *N. ceranae* and *N. apis* exhibited five and four distinct clades, respectively.

Reynali et al. showed that 51 out of 394 (13%) honey samples of *A. mellifera* from Argentina was positive for *A. apis* detection. Considering the phylogeny, this microbe was clustered into 6 patterns (A, B, C, D, E, and F). Also, pattern C was the most prevalent [95].

Considering SBV and *A. apis* infection, in this work, the number of *A. cerana* colonies infected by *A. apis* was 10 out of 50 (20%). Samples were from Chumphon province. In contrast, there was no infection by SBV in the collected bees. However, in Germany, the lower number of *A. mellifera* colonies infected by *A. apis* was 49 out of 1,277 (3.84%). SBV infection could be found in 4 out of 445 (0.90%) colonies [96].

Also, it was obvious that rate of infection depended on bee stage. In this research, the frequency of infection by nosema was lower in larvae comparing to adults. Also, infection by *A. apis* was more prevalent in larvae and pupae than in adults while the most prevalence of infection by *P. larvae* was in pupae. It was supported that, from total 46 samples collected in northern Thailand, 2 (4%) adults and 2 (4%) pupae of *A. mellifera* were infected by SBV. Nine (20%) adults and 21 (46%) pupae were infected by ABPV. Fifteen (33%) adults and 29 (63%) pupae were infected by DWV. One (2%) adult and 2 (4%) pupae were infected by KBV. From total 46 samples, 3 (6.5%) samples were infected by EFB and 2 (4.3%) samples were infected by chalkbrood [97].

Furthermore, very high rate of SBV infection in *A. cerana* was found in China at 86% for adults, 33% for pupae, and 79% for larvae, respectively [98]. In South Korea, the SBV infection in *A. cerana* was also very high [99]. Forty nine out of 81 adults (30.71%) and 137 out of 446 larvae (60.49%) were reported to be infected [100]. Especially *A. cerana* in Jeanla-do and Gyeongsang-do, 20 out of 26 adults (76.9%) and

31 out of 35 larvae (89.6%) were reported to get infected [99]. Furthermore, Chen et al. [90] showed that pupae of *A. cerana* were highly susceptible to get infected by *P. larvae* vegetative cells. On the contrary, 1-day-old larvae were the most susceptible to get infected by *P. larvae* spores.

In addition, Bamrick [101] revealed that the severity of infection depended on the larval instar and form of pathogen. Spores of *P. larvae* could highly infect intermediate larvae of *A. mellifera* (21 h old) at 100% and older larvae (45 h old) at 90%. Less ability of infection was found in younger larvae (3 h old) at 60% [101]. Moreover, it was reported that the vegetative cells of *P. larvae* could highly infect younger larvae of *A. mellifera* (77.5%) and intermediate larvae (75%) more than older larvae (55%). Moreover, Alippi and Aguilar [102] could isolate 3 groups of *P. larvae* (A, B, and C) from *A. mellifera* in Argentina and other countries by PCR and BOX primers [102]. It was also reported that 1-day-old larva could get infected by the much lower number of *P. larvae* spores (10 spores) than 2-days-old larva (millions of spores).

In order to find a safe way to kill those pathogens, probiotic is challenging. Thus, aerobic and anaerobic bacteria in healthy bee's gut were isolated and identified by the morphology of colony, the partial sequence of *16S rRNA*, and fermentation. The result was shown in Table 4.5 which *Bacillus* spp. was often obtained. After that, the antagonistic effect of these selected gut bacteria including *Bacillus* sp., *Pantoea* sp., *Azotobacter* sp., *Klebsiella* sp., and *Lactobacillus* sp. against strain 02 and strain 01 was determined by well diffusion. A diameter of inhibition zone was showed in Table 4.7. This finding was supported by Yoshiyama and Kimura [7] who found 35 bacteria isolated from the gut of *A. cerana japonica.* The large amount of gram positive and negative bacteria, especially *Bacillus* spp. was found and able to inhibit *P. larvae* growth by *in vitro* inhibition assay. Wu et al. [103] showed that eleven strains of *Bifidobacterium* sp. isolated from the gut of *A. cerana japonica* had the antagonistic effect against *Melissococcus plutonius* causing European foulbrood disease (EFB) by *in vitro* inhibition assay [103]. Moreover, Yoshiyama et al. [11] revealed that 9 strains such as *Enterococcus* sp., *Weissella* sp., and *Lactobacillus* sp. out of 208 LAB isolated from fermented materials could inhibit *P. larvae* growth by *in vitro* inhibition assay. Also, Jiang et al. [104] could isolate 14 strains of bacteria which were Firmicutes (5 strains),

Actinobacteria (2 strains), Alphaproteobacteria (3 strains), and Gammaproteobacteria (4 strains) from the gut of *A. cerana cerana* in China [104]. Besides, Sabaté et al. [105] reported 15 strains of *Bacillus* from the gut of *A. mellifera* (8 strains) and honey (7 strains) which 3 strains could inhibit the growth of *P. larvae* and *A. apis* by *in vitro* inhibition assay [105]. Also, Jeyaprakash et al. [106] revealed that *Bifidobacterium*, *Lactobacillus*, *Gluconacetobacter*, *Simonsiella*, *Serratia,* and *Bartonella* could be isolated from the worker gut of *A. mellifera capensis* and *A. mellifera scutellata* [106].

Olofsson and Vásquez could identify LAB flora in *A. mellifera* to be *Lactobacillus* and *Bifidobacterium* by PCR [107]. Then, it was classified into 4 clusters presenting *Lactobacillus* group, *Paenibacillus larvae* group, *Bifidobacterium* group, and *Pasteurellaceae* group. Evans and Armstrong [79] showed that 4 bacterial genera of *A. mellifera* larvae were *Acinetobacter*, *Bacillus*, *Brevibacillus*, and *Stenotrophomonas.* They could inhibit *P. larvae* growth with the inhibition zone larger than 40 mm. Later on, Porrini et al. found that the inhibition potential of *Bacillus* and *Enterococcus* isolated from *A. mellifera*'s mid gut and honey in Argentina [108] was from their metabolites which were surfactins and bacteriocins, respectively. Surfactin 2 had the antiparasitic activity which could inhibit the growth of microsporidian *N. ceranae*. That could extend the life of infected bees.

Besides inhibition by gut bacteria, antibiotics were tested. In this research, tetracycline, ampicillin, penicillin, streptomycin, and nalidixic acid were used. The result presented that different strains were sensitive to antibiotics differently. For strain 02, MIC values of those selected antibiotics were 0.3^{a} µg/ml while, for strain 01, MIC values were various (0.3^{a}, 0.3^{a}, 0.3^{a}, 0.6^{b}, and 0.7^{c} µg/ml, respectively). In overall, the range of clear zone was 0.9-1.4 cm. The data was supported by Alippi et al. [109]. It was reported that 27 out of 75 strains of *P. larvae* isolated from different geographic regions were efficiently inhibited by tetracycline [109]. The MIC value was in the range of 0.062-0.25 µg/ml and their clear zone was in the range of 3.33-7.01 cm. Besides, Okayama et al. reported that MIC of tetracycline and penicillin against 28 strains of *P. larvae* in Japan was in the range of 0.013-0.2 µg/ml and their clear zone was in the range of 1-26 mm [110].

Evans could isolate 4 haplotypes (A, B, C, and D) of *P. larvae* from Alberta (Canada), New York (US), South Dakota (US), and other states (North America). For haplotype A from Alberta, 48 out of 125 (38.4%) isolates could resist 5 μg of tetracycline while haplotype B from New York and South Dakota, 60 out of 125 (48%) isolates could resist 5 μg of tetracycline [111]. Not only different strains of pathogen were sensitive to any antibiotic variously, any pathogen was responded to many antibiotics differently as well. For example, *L. plantarum* (SH12 and SH24 strains) could stand 30 μg of tetracycline and 30 μg of kanamycin while it was sensitive to 10 μg of penicillin, 1 μg of oxacillin, 30 μg of vancomycin, 5 μg of rifampicin, and 2 μg of clindamycin [84].

Furthermore, Ozkirim et al. showed that *P. larvae* in Turkey could resist 10 μg of Sulbactam ampicillin (SAM) and Amoxycillin clavunolic acid (AMC) [112]. Pednekar et al. revealed that *Paenibacillus* sp. was resist to ampicillin (10 μg) and penicillin G (10 U) [113].

Normally, active metabolites were always secreted out of bacterial cells [108]. Thus, in this research, the supernatant of gut bacteria was used. Harsh condition like acidic, basic, highly temperature, was applied to the bacterial supernatant in order to reveal the stability of the active metabolites. The results showed that the supernatant from *Lactobacillus* sp., *Bacillus* sp., *Klebsiella* sp., *Azotobacter* sp., and *Pantoea* sp. could inhibit *P. larvae* growth although they were in those harsh conditions. The supernatant from *Lactobacillus* sp., *Klebsiella* sp., *Azotobacter* sp., and *Bacillus* sp. could still be active at 90 °C. In addition, the supernatant from *Lactobacillus* sp. could work efficiently at pH 8, 9, 10 while the supernatant from *Pantoea* sp. could work efficiently at pH 5.

Muhialdin et al. showed that the treated supernatant (pH 3 and 7) from *L. fermentum* and the treated supernatants (90 °C and 121 °C) from *L. fermentum* and *L. pentosus* could inhibit *Aspergillus niger* and *A. oryzae* growth [86]. Moreover, Magnusson and Schnurer reported that the treated supernatant (121 °C for 15 min, pH 3-4.5) from *L. coryniformis* subsp. *coryniformis* strain Si3 [114] could still inhibit the growth of molds (*A. fumigates*, *A. nidulans*, *Penicillium roqueforti*, *Mucor hiemalis*, *Talaromyces flavus*, *Fusarium poae*, *F. graminearum*, *F. culmorum*, and *F.*

sporotrichoides). Meantime, Torodov and Dicks was successful in identifying bacteriocin which was protein like compound in the supernatant from *L. plantarum* could inhibit gram negative bacteria growth [115]. It was active in the wide range of pH (2-12).

Moreover, pentocin was identified from the supernatant of *L. pentosus* and plantaricin was identified from the supernatant of *L. plantarum.* Pentocin [116] had the antimicrobial activity in the wide range of pH (1-10) but plantaricin [117] still had the antimicrobial activity at pH 1 and 9. Moreover, Muhialdin et al. revealed that pentocin from *L. pentosus* could show the antifungal activity at 121 °C for 1 h [86].

Besides, Oliveira et al. reported that the treated supernatant (pH 4-9 and 100 °C for 10 min) of LAB (*Lactococcus lactis*, *Pediococcus acidilactici*, *L. delbrueckii*, and *L. casei*) isolated from vacuum packaged beef could still have the antimicrobial activity [118].

In this work, the potential of targeted bacterial supernatants were totally affected by proteinase K and RNase A (data not shown). So, the antibacterial activity of the treated supernatant could not remain. Maurad and Meriem showed that the supernatant of *L. plantarum* lost the antibacterial activity totally after it was treated by proteinase K and chymotrypsin but it was partially inactive after treated with pronase E and trypsin [84]. Moreover, the antifungal activity of the supernatant of *L. coryniformis* was lost after it was treated by proteolytic enzymes such as proteinase K, trypsin, and pepsin [114]. In the contrary, Coconnier et al. reported that the supernatant of *L. acidophilus* could inhibit *Salmonella typhimurium* growth after it was treated by lactate dehydrogenase, trypsin, pronase, and proteinase K [119].

CHAPTER VI

CONCLUSIONS

1. For morphology investigation, random bees (larva, pupa, and adults) from 50 colonies were observed. Twelve percentage larvae from 6 colonies (15 samples) were attached by *Varroa* mites. Sixteen percentage larvae from 8 colonies (75 samples) looked black and brown. Thirty-two percentage pupae from 16 colonies (74 samples) were attached by *Varroa* mites. Four percentage pupae from 2 colonies (9 samples) looked black and brown. In contrast, no adult was attached by mites and abnormally looked.

2. Infected bees could be found in Samut-songkhram's and Chumphon's apiaries. The 20%, 6%, 4%, and 20% of all randomly selected samples were infected by *P. larvae*, *N. ceranae*, *N. apis*, and *A. apis*, respectively. In addition, larvae and pupae were more susceptible to infection than adults.

3. The partial sequences of amplified gene (*16S rRNA* for *P. larvae*, *N. ceranae*, and *N. apis*; *5.8S rRNA* for *A. apis*) were also used to construct phylogenetic trees by neighbor-joining algorithm (NJ plot). In overall, for each pathogen, they still had the close relationship within species of pathogen including *P. montaniterrae*, *N. bombycis*, *N. bombi*, and *B. bassiana*, respectively which were pathogen in silkworm and used as an outgroup. It indicated that there was no imported strain from outside. Thus, antibiotic or chemical resistance was still not a trouble.

4. In the future, phylogenetic analysis from *P. larvae*, *N. ceranae*, *N. apis*, and *A. apis* strains from different areas and dominant plant species may help to clarify the epidemiological presence in *A. cerana* from Samut-songkhram and Chumphon provinces, Thailand.

5. Bacteria in healthy bee's gut such as *Bacillus* sp., *Pantoea* sp., *Azotobacter* sp., *Staphylococcus* sp., *Klebsiella* sp., *Lactobacillus* sp., lactic acid bacteria, *Escherichia sp.*, and *Shigella* sp. were identified by the analyzed sequence of *16S rRNA*. Moreover, the same 30 colonies repeatedly identified by fermentation were *Bifidobacterium* sp., *Actinomyces* sp., *Photobacter* sp., *Klebsiella* sp., *Fusobacterium* sp., *Clostridium* sp., *Lactobacillus* sp., *Staphylococcus* sp., *Shigella* sp., and *Bacteroides* sp. Nonetheless, the same class of taxonomy could be obtained similarly by both methods.

6. The antagonistic effect of selected gut bacteria which were *Bacillus* sp., *Pantoea* sp., *Azotobacter* sp., *Klebsiella* sp., and *Lactobacillus* sp. against strain 02 and strain 01 was determined by well diffusion. It revealed that *Azotobacter* sp. showed the highest inhibitory effect on strain 02 with the diameter of relative inhibition zone at 56.68^{c} ± 3.17%. The variance difference was significant at the 0.05 level (*p*-value: 0.000).

7. For strain 02, the same MIC value of streptomycin and nalidixic acid was 0.3[a] µg/ml. In addition, for strain 01, the MIC value of streptomycin was 0.6[b] µg/ml and the MIC value of nalidixic acid was 0.7[c] µg/ml, respectively. The evaluation at MIC value of strain 01 exhibited higher antibiotic resistance properties than strain 02.

8. The supernatant of targeted bacteria was also tested for inhibitory effect on the growth of strain 01 and strain 02. It showed that the antibacterial activity against strain 01 and strain 02 at 37 °C were from the supernatant of *Bacillus* sp., *Lactobacillus* sp., *Klebsiella* sp., and *Azotobacter* sp. In addition, the supernatant of *Lactobacillus* sp. revealed the highest inhibitory activity which the mean difference was significant at the 0.05 level (*p*-value: 0.010).

9. The supernatant of each probiotic bacteria treated at 90 °C for 1 h was also tested for the inhibitory effect on the growth of strain 01 and strain 02. In overall, the treated supernatant from *Lactobacillus* sp. (no growth), *Klebsiella* sp. (12.48[a] ± 1.06%), *Azotobacter* sp. (8.75[a] ± 2.10%), and *Bacillus* sp. (6.41[a] ± 7.44%) revealed the highest inhibitory activity which the mean difference was not significant at the 0.05 level (*p*-value: 0.238).

10. All supernatants of isolated probiotic treated at 121 °C for 1 h or treated with proteinase K and RNase A did not show the inhibitory effect against strain 01 and strain 02 at all. At 72 h incubation, the percentage of strain 01 and strain 02 growth still increased, comparing to positive control.

11. In overall, the supernatant from *Pantoea* sp. at pH 5 (no growth for strain 01 and $5.45^{a} \pm 1.34\%$ for strain 02), *Bacillus* sp. at pH 3 ($23.48^{a} \pm 13.93\%$ for strain 01 and $10.92^{a} \pm 17.38\%$ for strain 02), and *Klebsiella* sp. at pH 3 ($45.04^{a} \pm 15.27\%$ for strain 01) revealed the highest inhibitory activity which the mean difference was not significant at the 0.05 level (*p*-value: 0.238).

12. Furthermore, the treated supernatant from *Lactobacillus* sp. at pH 8 ($532.67^{a} \pm 44.96\%$ for strain 01), and pH 10 ($536.15^{a} \pm 22.83\%$ for strain 01), and *Klebsiella* sp. at pH 10 ($529.15^{a} \pm 45.91\%$ for strain 01) revealed the highest inhibitory activity which the mean difference was not significant at the 0.05 level (*p*-value: 1.000).

13. Perspective and application from this research was that we could get gut bacteria in healthy bee which could inhibit the growth of bee pathogens. In the future, those probiotic can be used in pathogen control and food industry.

REFERENCES

1. Moritz, R., et al., *Research strategies to improve honeybee health in Europe.* Apidologie, 2010. **41**: p. 227-242.
2. Peng, Y., et al., *Response of foster Asian honeybee (Apis cerana Fabr.) colonies to the brood of European honeybee (Apis mellifera L.) infested with parasitic mite, Varroa jacobsoni oudemans.* Journal of Invertebrate Pathology, 1987. **49**: p. 259-264.
3. Piccini, C., et al., *Detection of Paenibacillus larvae subspecies larvae spores in naturally infected bee larvae and artificially contaminated honey by PCR.* Journal of Microbiology and Biotechnology, 2002. **18**: p. 761-765.
4. Hamiduzzaman, M., E. Guzman, and P. Goodwin, *A multiplex PCR assay to diagnose and quantify Nosema infections in honeybees (Apis mellifera).* Journal of Invertebrate Pathology, 2010. **105**: p. 151-155.
5. Yang, J.L., et al., *Rapid detection of sacbrood virus (SBV) by one-step reverse transcription loop-mediated isothermal amplification assay.* Virology Journal, 2012. **9**: p. 47.
6. James, R.R. and J.S. Skinner, *PCR diagnostic methods for Ascosphaera infections in bees.* Journal of Invertebrate Pathology, 2005. **90**(2): p. 98-103.
7. Yoshiyama, M. and K. Kimura, *Bacteria in the gut of Japanese honeybee, Apis cerana japonica, and their antagonistic effect against Paenibacillus larvae, the causal agent of American foulbrood.* Journal of Invertebrate Pathology, 2009. **102**(2): p. 91-96.
8. Fukuda, S., et al., *Bifidobacteria can protect from enteropathogenic infection through production of acetate.* Nature, 2011. **469**: p. 543-547.
9. Nikoskelainen, S., et al., *Immune enhancement in rainbow trout (Oncorhynchus mykiss) by potential probiotic bacteria (Lactobacillus rhamnosus).* Fish and Shellfish Immunology, 2003. **15**: p. 443-452.
10. Evans, J.D. and D.L. Lopez, *Bacterial probiotics induce an immune response in the honeybee (Hymenoptera: Apidae).* Journal of Economic Entomology, 2004. **97**: p. 752-756.
11. Yoshiyama, M., et al., *Inhibition of Paenibacillus larvae by lactic acid bacteria isolated from fermented materials.* Journal of Invertebrate Pathology, 2013. **112**(1): p. 62-67.

12. Fabricius, J.C., *Entomologia systematica emendata et aucta.* Secundum classes, ordines, genera, species adjectus synonymis, locis, observationibus, descriptionibus, 1793. **C. G. Proft**: p. Copenhagen.
13. Smith, F., *Catalogue of the hymenopterous insects collected at Sarawak, Borneo; Mount Ophir, Malacca; and at Singapore, by AR Wallace.* Journal of the Proceedings of the Linnean Society of London, 1858. **2**: p. 42-130.
14. Linnaeus, C., *Systema naturae per regna tria naturae.* Secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis, 1758. **Laurentii Salvii**: p. Stockholm.
15. Tingek, S.M., et al., *Rediscovery of Apis vechti (Maa, 1953): the Saban honey bee.* Apidologie, 1988. **19**: p. 97-102.
16. Engel, M.S., *Fossil honeybees and evolution in the genus Apis (Hymenoptera: Apidae).* Apidologie, 1998. **29**: p. 265-281.
17. Wongsiri, S., et al., *Efficiency of crossing the Chinese strain Apis cerana cerana with the Thai strain A. cerana indica (Apidae: Hymenoptera) by artificial insemination.* Honeybee Science, 1989. **10**: p. 112-119.
18. Skorikov, A.S., *Eine neue Basis für eine Revision der Gattung Apis L.* Report on Applied Entomology, Liningrad, 1929. **4**: p. 249-270.
19. Engel, M.S., *The taxonomy of recent and fossil honeybees (Hymenoptera: Apidae; Apis).* Journal of Hymenoptera Research, 1999. **8**: p. 165-196.
20. Enderlein, G., *Neue Honigbienen und Beiträge zur Kenntnis der Verbreitung der Gattung Apis.* Stettiner Entomologische Zeitung 67: 331–344., 1906. **67**: p. 331-344.
21. Ruttner, F., J. Woyke, and N. Koeniger, *Reproduction in Apis cerana. 2. Reproductive organs and natural insemination.* Journal of Agricultural Research, 1973. **12**: p. 21-34.
22. Chantawannakul, P., S. Petersen, and S. Wongsiri, *Conservation of honeybee species in South East Asia: Apis mellifera or native bees.* Biodiversity, 2004. **5**: p. 25-28.
23. Dyer, F.C. and T.D. Seeley, *Nesting behavior and the evolution of worker tempo in four honeybee species.* Ecology, 1991. **72**: p. 156-171.
24. Woodrow, A.W., *Behavior of honeybees towards brood infected with American foulbrood.* American Bee Journal, 1941. **81**: p. 363-366.
25. Oldroyd, B.P., et al., *Task specialization in a wild bee, Apis florea (Hymenoptera: Apidae), revealed by RFLP banding.* Behavior Ecology Sociobiology 1994. **34**: p. 25-30.

26. Jacob, A., P.A. Rajan Asari, and N. Mohandas, *A new disease of the Indian honeybee, Apis cerana indica F. in Kerala.* Agricultural Research Journal Kerala, 1992. **30**: p. 63-64.
27. Chantawannakul, P. and B.N. Dancer, *American foulbrood in honeybees.* Bee World, 2001. **82**: p. 168-180.
28. Genersch, E., *American Foulbrood in honeybees and its causative agent, Paenibacillus larvae.* Journal of Invertebrate Pathology, 2010. **103, Supplement**(0): p. S10-S19.
29. Haseman, L., *How long can spores of American foulbrood live?* American Bee Journal, 1961. **101**: p. 289-299.
30. Alippi, A.M., et al., *In vitro and in vivo susceptibility of the honeybee bacterial pathogen Paenibacillus larvae subsp. larvae to the antibiotic tylosin.* Veterinary Microbiology, 2005. **109**(1–2): p. 47-55.
31. Dancer, B.N. and P. Chantawannakul, *The Proteases of American Foulbrood Scales.* Journal of Invertebrate Pathology, 1997. **70**(2): p. 79-87.
32. Ho, K. and Y. Chen, *Susceptibility of the Asian honeybee (Apis cerana) to American foulbrood (Paenibacillus larvae larvae).* Journal of Apicultural Research, 2001. **39**: p. 169-175.
33. Bamrick, J.F. and W.C. Rothenbuhler, *Resistance to American foulbrood in honeybees. IV. The relationship between larval age at inoculation and mortality in a resistant and in a susceptible line.* Journal of Insect Pathology, 1961. **3**: p. 381-390.
34. Davidson, E.W., *Ultrastructure of American foulbrood disease pathogenesis in larvae of the worker honey bee, Apis mellifera.* Journal of Invertebrate Pathology, 1973. **21**(1): p. 53-61.
35. Rinderer, T.E., W.C. Rothenbuhler, and T.A. Gochnauer, *The influence of pollen on the susceptibility of honey-bee larvae to Bacillus larvae.* Journal of Invertebrate Pathology, 1974. **23**(3): p. 347-350.
36. Danka, R.G. and J.D. Villa, *Preliminary observations on the susceptibility of Africanized honeybees to American foulbrood.* Journal of Apicultural Research 33: 342-245., 1994. **33**: p. 243-245.
37. Human, H., et al., *The honeybee disease American foulbrood - An African perspective.* African Entomology, 2011. **19**: p. 551-557.
38. Holst, E.C., *Newer knowledge of American foulbrood.* Gleanings in Bee Culture, 1946. **74**: p. 138-139.

39. Dobbelaere, W., D.C. Graaf, and J.E. Peeters, *Development of a fast and reliable diagnostic method for American foulbrood disease (Paenibacillus larvae subsp. larvae) using a 16S rRNA gene based PCR.* Apidologie, 2001. **32**: p. 363-370.
40. Bailey, L., B.V. Ball, and J.N. Perry, *The prevalence of viruses of honeybees in Britain.* Annals of Applied Biology, 1981. **97**: p. 109-118.
41. Bailey, L., J.M. Carpenter, and R.D. Woods, *A strain of sacbrood virus from Apis cerana.* Journal of Invertebrate Pathology, 1982. **39**(2): p. 264-265.
42. Kilwinski, J., et al., *Proposal to reclassify Paenibacillus larvae subsp. pulvifaciens DSM 3615 (ATCC 49843) as Paenibacillus larvae subsp. larvae. Results of a comparative biochemical and genetic study.* Veterinary Microbiology, 2004. **104**(1–2): p. 31-42.
43. Antúnez, K., et al., *Paenibacillus larvae larvae spores in honey samples from Uruguay: a nationwide survey.* Journal of Invertebrate Pathology, 2004. **86**(1–2): p. 56-58.
44. Gojmerac, W.L., *Bees, beekeeping, honey and pollination.* Avi Publishing Company. U.S.A., 1980.
45. Gochnauer, T.A. and B. Furgala, *Chemotherapy of Nosema disease further studies on the interaction of fumagillin and the antibacterial agents, sodium sulfathiazole and oxytetracycline.* American Bee Journal, 1981. **121**: p. 253-254.
46. Bailey, L., B.V. Ball, and J.N. Perry, *Association of viruses with two protozoal pathogens of the honeybee.* Annals of Applied Biology, 1983. **103**: p. 13-20.
47. Chen, Y.P. and R. Siede, *Honey Bee Viruses*, in *Advances in Virus Research*, A.J.S. Karl Maramorosch and A.M. Frederick, Editors. 2007, Academic Press. p. 33-80.
48. Whitaker, J., A. Szalanski, and M. Kence, *Molecular detection of Nosema ceranae and N. apis from Turkish honeybees.* Apidologie, 2010. **42**: p. 174-180.
49. Gilliam, M., *Identification and roles of non-pathogenic microflora associated with honey bees.* FEMS Microbiology Letters, 1997. **155**(1): p. 1-10.
50. Spiloir, C.F. and L.S. Olive, *A reclassification of the genus Pericystis Betts.* Mycologia, 1955. **47**: p. 238-244.
51. Maghrabi, H.A. and L.P. Kish, *Isozyme characterization of Ascosphaerales associated with bees. IV. Analyses.* Mycologia 1987. **79**: p. 519-523.
52. Theantana, T. and P. Chantawannakul, *Protease and β-N-acetylgluco saminidase of honey bee chalkbrood pathogen Ascosphaera apis.* Journal of Apicultural Research, 2008. **47**: p. 68-76.

53. Maghrabi, H.A. and L.P. Kish, *Isozyme characterization of Ascosphaerales associated with bees. II. Ascosphaera major, A. atra and A. asterophora.* Mycologia, 1985. **77**: p. 366-372.
54. Aronstein, K.A. and K.D. Murray, *Chalkbrood disease in honey bees.* Journal of Invertebrate Pathology, 2010. **103, Supplement**(0): p. S20-S29.
55. Lu, R., D.W. Goerzen, and G.H. Rank, *Use of RAPD Analysis for in Situ Identification of Ascosphaera aggregata and Ascosphaera larvisin Larval Cadavers of the Alfalfa Leafcutting Bee, Megachile rotundata.* Journal of Invertebrate Pathology, 1996. **68**(1): p. 78-83.
56. Bissett, J.D., G.M. Duke, and M.S. Goettel, *Ascosphaera acerosa sp. nov. isolated from the alfalfa leafcutting bee, with a key to the species of Ascosphaera.* Mycologia, 1996. **88**: p. 797-803.
57. Betts, A.D., *Fungus diseases of bees.* Bee World, 1932. **1**: p. 132.
58. Seal, D.W.A., *Chalkbrood disease of bees.* New Zealand Journal of Agriculture, 1957. **6**: p. 562.
59. Health, L.A.F., *Occurrence and distribution of chalkbrood disease of honeybees.* Bee World, 1985. **66**: p. 9-15.
60. Chantawannakul, P., et al., *A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in Varroa mites collected from a Thai honeybee (Apis mellifera) apiary.* Journal of Invertebrate Pathology, 2007. **91**: p. 69-73.
61. Taber, S., R. Sacrett, and J. Mills, *A possible control for chalkbrood disease.* American Bee Journal, 1975. **115**: p. 20.
62. Gochnauer, T.A., P.W. Burke, and J. Benazet, *Large-scale fumigation with ethylene oxide of honeybee combs contaminated with Bacillus larvae.* Journal of Apicultural Research, 1979. **18**: p. 302-308.
63. Gochnauer, T.A., B. Furgala, and H. Shimanuki, *Diseases and enemies of the honeybee.* The Hive and the Honey Bee, 1975. **Illionion**: p. 651-661.
64. Calderone, N.W. and H. Shimanuki, *An in vitro evaluation of botanical compounds for the control of the honeybee pathogens Bacillus larvae and Ascosphaera apis, and the secondary invader B. alvei.* Journal of Essential Oil Research, 1994. **6**: p. 279-287.
65. Maghrabi, H.A. and L.P. Kish, *Isozyme characterization of Ascosphaerales associated with bees. I. Ascosphaera apis, A. proliperda and A. aggregata.* Mycologia, 2001. **77**: p. 358-365.

66. Anderson, D.L. and A.J. Gibbs, *Transpuparial transmission of Kashmir bee virus and sacbrood virus in the honeybee Apis mellifera.* Annals of Applied Biology, 1989. **1141**: p. 1-7.
67. Joshi, N.K. and S.K. Verma, *Studies on sacbrood disease in Apis cerana indica Fab. in Kumaon hills of UP., India.* Indian Bee Journal, 1985. **47**: p. 41-42.
68. White, G.F., *The cause of European foulbrood. Circular,.* US Bureau of Entomology, 1912. **157**.
69. Chen, Y., et al., *Multiple virus infections in the honeybee and genome divergence of honey bee viruses.* Journal of Invertebrate Pathology, 2004. **87**: p. 84-93.
70. Bradbear, N., *World distribution of major honeybee diseases and pests.* Bee World, 1988. **69**: p. 15-39.
71. Shah, F.A. and T.A. Shah, *Thai sacbrood disease of Apis cerana.* Indian Bee Journal, 1988. **50**: p. 110-112.
72. Aronstein, K., et al., *High mobility group (HMG-box) genes in the honeybee fungal pathogen Ascosphaera apis.* Mycologia, 2007. **99**: p. 553-561.
73. Chen, Y.P., et al., *Morphological, molecular, and phylogenetic characterization of Nosema ceranae, a microsporidian parasite isolated from the European honeybee, Apis mellifera.* Journal of Eukaryotic Microbiology, 2009. **56**: p. 142-147.
74. Alippi, A.M., A.C. López, and O.M. Aguilar, *Differentiation of Paenibacillus larvae subsp. larvae, the cause of American foulbrood of honeybees, by using PCR and restriction fragment analysis of genes encoding 16S rRNA.* Applied Environmental Microbiology 2002. **68**: p. 3655-3660.
75. Grabensteiner, E., et al., *Sacbrood virus of the honeybee (Apis mellifera): Rapid identification and phylogenetic analysis using reverse transcription-PCR.* Clinical and Diagnostic Laboratory Immunology, 2001. **8**: p. 93-104.
76. Yoshiyama, M. and K. Kimura, *Distribution of Nosema ceranae in the European honeybee, Apis mellifera in Japan.* Journal of Invertebrate Pathology, 2011. **106**(2): p. 263-267.
77. Alippi, A.M. and F.J. Reynaldi, *Inhibition of the growth of Paenibacillus larvae, the causal agent of American foulbrood of honeybees, by selected strains of aerobic spore-forming bacteria isolated from apiarian sources.* Journal of Invertebrate Pathology, 2006. **91**(3): p. 141-146.
78. Forsgren, E., et al., *Novel lactic acid bacteria inhibiting Paenibacillus larvae in honeybee larvae.* Apidologie, 2010. **41**: p. 99-108.

79. Evans, J.D. and T.N. Armstrong, *Antagonistic interactions between honeybee bacterial symbionts and implications for disease.* BMC Ecology, 2006. **6**: p. 1-9.
80. Reyes, M.G., et al., *In vitro inhibition of Paenibacillus larvae by different extracts and pure compounds from Flourensia spp.* Industrial Crops and Products, 2013. **50**(0): p. 758-763.
81. Santos, R.C.V., et al., *Antimicrobial activity of Amazonian oils against Paenibacillus species.* Journal of Invertebrate Pathology, 2012. **109**(3): p. 265-268.
82. Stevens, K.A., B.W. Sheldon, and N.A. Klapes, *Nisin treatment for inactivation of Salmonella species and other gram-negative bacteria.* Applied Environmental Microbiology, 1991. **57**: p. 3613-3615.
83. Ahmed, Z., et al., *Lactobacillus acidophilus bacteriocin, from production to their application: An overview.* African Journal of Biotechnology, 2010. **9**: p. 2843-2850.
84. Maurad, K. and K.H. Meriem, *Probiotic characteristics of Lactobacillus plantarum strains from traditional butter made from camel milk in arid regions (Sahara) of Algeria.* Grasas Y Aceites, 2008. **59**: p. 218-224.
85. Jatikavanich, S. and K. Juntongjin, *General Microbiology Laboratory.* Chulalongkorn University Printing House, Bangkok, 1996.
86. Muhialdin, B.J., et al., *Effect of pH and heat treatment on antifungal activity of Lactobacillus fermentum Te007, L. pentosus G004 and Pediococcus pentosaceus Te010.* Innovative Romanian Food Biotechnology, 2011. **8**: p. 41-53.
87. Aween, M.M., et al., *Antibacterial activity of Lactobacillus acidophilus strains isolated from honey marketed in Malaysia against selected multiple antibiotic resistant (MAR) gram-positive bacteria.* Journal of Food Science, 2012. **77**: p. 364-371.
88. Han, S.-H., et al., *Ultra-rapid real-time PCR for the detection of Paenibacillus larvae, the causative agent of American Foulbrood (AFB).* Journal of Invertebrate Pathology, 2008. **99**(1): p. 8-13.
89. Lauro, F.M., et al., *Rapid detection of Paenibacillus larvae from honey and hive samples with a novel nested PCR protocol.* International Journal of Food Microbiology, 2003. **81**(3): p. 195-201.
90. Chen, Y.W., et al., *Susceptibility of the Asian honeybee, Apis cerana to American foulbrood, Paenibacillus larvae larvae.* Journal of Apicultural Research, 2000. **39**: p. 169-175.
91. Chaimanee, V., N. Warrit, and P. Chantawannakul, *Infections of Nosema ceranae in four different honeybee species.* Journal of Invertebrate Pathology, 2010. **105**(2): p. 207-210.

92. Chen, Y., et al., *Asymmetrical coexistence of Nosema ceranae and Nosema apis in honey bees.* Journal of Invertebrate Pathology, 2009. **101**(3): p. 204-209.
93. Botías, C., et al., *Further evidence of an oriental origin for Nosema ceranae (Microsporidia: Nosematidae).* Journal of Invertebrate Pathology, 2012. **110**: p. 108-113.
94. Chaimanee, V., et al., *Phylogenetic analysis of Nosema ceranae isolated from European and Asian honeybees in Northern Thailand.* Journal of Invertebrate Pathology, 2011. **107**(3): p. 229-233.
95. Reynaldi, F.J., et al., *Differentiation of Ascosphaera apis isolates by rep-PCR fingerprinting and determination of chalkbrood incidence in Argentinean honey samples.* Journal of Apicultural Research, 2003. **42**: p. 68-76.
96. Hedtke, K., et al., *Evidence for emerging parasites and pathogens influencing outbreaks of stress-related diseases like chalkbrood.* Journal of Invertebrate Pathology, 2011. **108**(3): p. 167-173.
97. Sanpa, S. and P. Chantawannakul, *Survey of six bee viruses using RT-PCR in Northern Thailand.* Journal of Invertebrate Pathology, 2009. **100**(2): p. 116-119.
98. Ai, H., X. Yan, and R. Han, *Occurrence and prevalence of seven bee viruses in Apis mellifera and Apis cerana apiaries in China.* Journal of Invertebrate Pathology, 2012. **109**(1): p. 160-164.
99. Choe, S.E., et al., *Prevalence and distribution of six bee viruses in Korean Apis cerana populations.* Journal of Invertebrate Pathology, 2012. **109**(3): p. 330-333.
100. Choe, S.E., et al., *Genetic and phylogenetic analysis of South Korean sacbrood virus isolates from infected honeybees (Apis cerana).* Veterinary Microbiology, 2012. **157**: p. 32-40.
101. Bamrick, J.F., *Resistance to American foulbrood in honey bees: VI. Spore germination in larvae of different ages.* Journal of Invertebrate Pathology, 1967. **9**(1): p. 30-34.
102. Alippi, A.M. and O.M. Aguilar, *Characterization of Isolates of Paenibacillus larvae subsp. larvae from Diverse Geographical Origin by the Polymerase Chain Reaction and BOX Primers.* Journal of Invertebrate Pathology, 1998. **72**(1): p. 21-27.
103. Wu, M., et al., *Characterization of bifidobacteria in the digestive tract of the Japanese honeybee, Apis cerana japonica.* Journal of Invertebrate Pathology, 2013. **112**(1): p. 88-93.
104. Jiang, W.-y., et al., *The Influence of Bt-Transgenic Maize Pollen on the Bacterial Diversity in the Midgut of Chinese Honeybees, Apis cerana cerana.* Journal of Integrative Agriculture, 2013. **12**(3): p. 474-482.

105. Sabaté, D.C., L. Carrillo, and M. Carina Audisio, *Inhibition of Paenibacillus larvae and Ascosphaera apis by Bacillus subtilis isolated from honeybee gut and honey samples.* Research in Microbiology, 2009. **160**(3): p. 193-199.

106. Jeyaprakash, A., M.A. Hoy, and M.H. Allsopp, *Bacterial diversity in worker adults of Apis mellifera capensis and A. mellifera scutellata (Insecta: Hymenoptera) assessed using 16S rRNA sequences.* Journal of Invertebrate Pathology, 2003. **84**: p. 96-103.

107. Olofsson, T.C. and A. Vásquez, *Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee Apis mellifera.* Microbiology, 2008. **57**: p. 356–363.

108. Porrini, M.P., et al., *Effect of bacterial metabolites on microsporidian Nosema ceranae and on its host Apis mellifera.* Parasitology Research, 2010. **107**: p. 381-388.

109. Alippi, A.M., et al., *Evidence for plasmid-mediated tetracycline resistance in Paenibacillus larvae, the causal agent of American Foulbrood (AFB) disease in honeybees.* Veterinary Microbiology, 2007. **125**(3–4): p. 290-303.

110. Okayama, A., et al., *Biological properties and antibiotic susceptibility of Bacillus larvae originated from American foulbrood of honeybee in Japan.* Journal of Veterinary Medical Science, 1996. **58**: p. 439-441.

111. Evans, J.D., *Diverse origins of tetracycline resistance in the honey bee bacterial pathogen Paenibacillus larvae.* Journal of Invertebrate Pathology, 2003. **83**(1): p. 46-50.

112. Ozkirim, A., S. Aktaş, and N. Keskin, *Screening alternative antibiotics against Paenibacillus larvae subsp. larvae strains isolated from the different regions of Turkey.* Mellifera 2007. **7**: p. 2-8.

113. Pednekar, P.B., et al., *Isolation of multi-drug resistant Paenibacillus sp. from fertile soil: An imminent menace of spreading resistance.* Journal of Life Sciences, 2010. **4**: p. 7-12.

114. Magnusson, J. and J. Schnürer, *Compound broad-spectrum proteinaceous antifungal Lactobacillus coryniformis subsp. coryniformis strain Si3 produces a broad-spectrum proteinaceous antifungal compound.* Applied Environmental Microbiology, 2001. **67**: p. 1-5.

115. Todorov, S.D. and L.M.T. Dicks, *Lactobacillus plantarum isolated from molasses produces bacteriocins active against Gram-negative bacteria.* Enzyme and Microbial Technology, 2005. **36**(2–3): p. 318-326.

116. Okkers, D.J., et al., *Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from Lactobacillus pentosus with a fungistatic effect on Candida albicans.* Journal of Applied Microbiology, 1999. **87**: p. 726-734.
117. Hernández, D., E. Cardell, and V. Zárate, *Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: Initial characterization of plantaricin TF711, a bacteriocin-like substance produced by Lactobacillus plantarum TF711.* Journal of Applied Microbiology, 2005. **99**: p. 77-84.
118. Oliveira, R.B.P., A.L. Oliveira, and M.B.A. Glória, *Screening of lactic acid bacteria from vacuum packaged beef for antimicrobial activity.* Brazilian Journal of Microbiology, 2007. **39**: p. 368-374.
119. Coconnier, M.H., et al., *Antibacterial effect of the adhering human Lactobacillus acidophilus strain LB.* Antimicrobial Agents and Chemotherapy, 1998. **41**: p. 1046-1052.

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